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13. ABSTRACT ***(Maximum 200 Words)***

The original goal was to purify both NK cell receptor molecules (Ly49C) and cognate class I MHC ligands (H2-K^b) to determine the crystal structure of Ly49C bound to K^b. Others using Ly49A and H2-D^d solved this aim Therefore we chose to test the effect of 'blocking' receptor-class I interactions on kill ing of tumor cells by host NK cells. Using 5E6 monoclonal antibody F(ab')$_2$ fragments against inhibitory receptors Ly49C and I of NK cells, we observed enhanced lysis of H2-K^b syngeneic tumor cells (EL-4 and C1498). In vivo, C14998 leukemia treated with 5E6 F(ab')$_2$ in C57BL/6 mice increased survival times with 25% cures. The same treatment enhanced the ability of mice to 'clear' radiolabeled tumor cells from lungs of mice. Activation of NK cells with interleukin 2 (IL2) in vitro, followed by infusion into mice previously challenged with C1498 tumor cells, produced an even greater treatment effect (50-60% cures). Finally, mixtures of C1498 cells, C57BL/6 marrow cells and NK cells treated (or not) with 5E6 F(ab')$_2$ fragments in the presence of IL2, were infused into irradiated syngeneic host mice. The NK cells were able to 'purge' the tumor cells so that leukemia was decreased in incidence in the recipient mice.

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Introduction

Natural killer cells lyse tumor cells, virally infected cells, autologous T cells that lose expression of class I antigens, and even autologous dendritic cells that express normal levels of class I antigens (1-3). Based on the findings that F1 hybrid mice reject parental strain marrow cell grafts (4) and that class I deficient tumor cells are exquisitly susceptible to NK cell lysis, Ljunggren and Kaerre developed the "missing self' hypothesis (5). They predicted that some NK cell receptor would recognize a ligand on prospective target cells that would prevent rather than induce a signal to kill the cell. Yokoyama discovered the Ly49 genes and described the NK gene complex on chromosome 6 of mice (6,7). These genes encoded lectin-like receptors that were homodimers and recognized class I molecules. The original plan of this project was to crystallize the complex of Ly49C and H2-K^b, but others succeeded in doing that with a similar NK receptor (Ly49A) and class I MHC molecule (H2-D^d) complex (8). Initially only inhibitory receptors were detected, but eventually positive signaling Ly49 receptors were discovered that lack the inhibitory motifs in the cytoplasmic domains. These receptors associate with DAP12, a cytoplasmic adaptor protein that bears an activating motif, using a salt bridge in the transmembrane region (9). Our group presented evidence that NK cell lysis of H2-D^{d+} tumor cells in vitro and rejection of marrow grafts in vivo were governed by the presence of activating Ly49D receptors and presence or absence of inhibitory Ly49G2, A or C receptors (10, 11). These and other data led to the hypothesis that NK cell anti-syngeneic (autologous) leukemia effects should be greatly enhanced if one could block negative signals to class I antigens. Antibodies to the class I antigens or to the Ly49 receptors do enhance lysis in vitro (12). However, because so many cells express class I in the body, we chose to use the monoclonal antibodies (mAb) to Ly49 inhibitory receptors. If we treat C57BL/6 (B6) mice bearing syngeneic C1498 leukemia cells with non-depleting antibody $F(ab')_2$ fragments to NK Ly49I and C inhibitory receptors, survival is enhanced. This reagent allows B6 NK cells stimulated with IL-2 to 'purge' leukemia cells from marrow cell suspensions. The transfer of these purged marrow cells to irradiate mice enhances survival relative to the transfer of similar cells without treatment with the blocking reagent.

Body

During the first 12 months, Charles Haseman, Ph. D., developed the reagents for the crytallizaton of Ly49C and H2-K^b. During this time, a group at NIH led by David Morgulis solved the crystal structure of Ly49A bound to H2-D^d class I MHC molecule (8). The interaction between Ly49A and Dd is much stronger than with any other Ly49-class I MHC molecules, according to Dr. Margulis (personal communication). He has now worked with other Ly49 receptor molecules, and has observed that they differ in affinity for relevant class I molecules and in other ways.

Our group has worked on the use of the knowledge gained in studies of Ly49 inhibitory receptors to develop a new method to treat leukemia, i.e. block negative signals to NK cells so that the activating receptors can act unimpeded from those inhibitory receptors. As described in the last annual report, we have observed that blocking 5E6 (Ly49C and I) receptors with $F(ab')_2$ anti-5E6 mAbs enhances the lysis of syngeneic tumor C1498 or EL-4 leukemia or lymphomas cells by B6 NK cells (Figure 1) in a ^{51}Cr-release assay. Flow cytometry indicated that both whole and $F(ab')_2$ 5E6 mAbs stain about 30% of B6 NK1.1+ (gated) NK cells (Figure 1).

The second test of these blocking reagents employed an in vitro tumor colony growth assay, using C1498 and El-4 cells of B6 strain mice, and P815 H2d mastocytoma tumor cells. In this

experiment, we utilized two F(ab')$_2$ mAb - 5E6 and 4D11. 4D11 reacts with Ly49G2 recepotrs that receive negative signals primarily from H2-D^d. B6 severe combined immunodeficient (SCID) mice lacking both B and T cells were donors of spleen cells that were activated in vitro with rhIL-2 for 5-7 days. These cells were treated with 25 μg normal IgG, 5E6 F(ab')$_2$ or 4D11 F(ab')$_2$ mAbs for 3 hours. At this time, small numbers of tumor cells were added to each well and the mixture was incubated for 2 days to allow time for NK cells to lyse the tumor cells. The cells were transferred to a semi-solid medium and cultured for 5-7 days to allow tumor colonies to develop. Tumor cells with any of the reagents used had some anti-tumor effect (Figure 2), but the 5E6 reagent was superior to the two others against the H2b C1498 and EL-4 tumor cells. Both 4D11 and 5E6 were superior to IgG controls when tested against H2d P815 cells. This result suggests that multiple blocking antibody reagents may amplify the anti-tumor effects of NK cells.

The critical test of this approach is in vivo anti-tumor effects. The first type of experiment, B6 mice were injected with 5E6 F(ab')2 reagent 2 days before and twice weekly for 3 weeks after challenge with 30,000 C1498 leukemia cells. Survival was prolonged and about 35% of mice were cured, $p = 0.0034$ (Figure 3). The second type of experiment involved the use of NK cells to 'purge' C1498 cells from suspensions of mixtures of NK and C1498 cells in the presence of 5E6 or 4D11 or normal mouse serum. The mixture of cells was infused into B6 mice. This was followed on days 18 and 25 by transfer of NK cells activated in vitro with IL-2 in the presence of the same reagents. Mice received 50,000 units of rhIL-2 i.p. to maintain function of the NK cells in vivo for several days. This treatment approach was very successful, resulting in about 50% cures and prolongation of life if 5E6 F(ab')$_2$ was used (Figure 3). There were anti-leukemia effects when NK cells were used and either normal mouse serum or 4D11 F(ab')$_2$ was used. This work has now been published (13) and is in the appendix.

We are developing imaging methods to analyze anti-leukemia effects in vivo. The Radiological Science group at this University is developing imaging methods that are adapted to use of small experimental animals. We want to rapidly visualize anti-tumor effects and to detect mechanisms of cell death of the targeted tumor cells. The goal is to develop rapid assays that will analyze the ability of these blocking antibodies to augment anti-leukemia effects as early as possible after infusion of the leukemia cells. This includes the very early step of clearance of radiolabeled tumor cells from the lung (14). In figure 4 we show images of a mouse infused with ^{111}InOxine-labeled YAC-1 tumor cells that are very sensitive to NK cell lysis. At 15 min. localization is restricted to the lungs. By 3 hours, the lungs contain much less of the ^{111}In, while the liver/spleen area has now gained radioactivity. In addition, a ^{99m}Tc agent that goes to liver/spleen but not to lung was infused just before the 3 hour point. The overlay of the two isotopes (green and red) produced a yellow area over the liver/spleen. Counts of the lung, spleen and liver removed from the animal after sacrifice mirrored the data obtained by imaging. This method could be used to test for relative rate of tumor cell killing in mice injected with blocking antibodies. The imaging method can also be used to determine the mechanism of cell killing by using radiolabeled molecules that detect dying cells undergoing apoptosis versus necrosis.

During the last 12 months of this grant period, we have extended the work described above. In a second (submitted) publication (15), we describe a study of the ability of NK cells to 'purge' C1498 leukemia cells from a marrow cells suspension and to test the effect of NK cells on syngeneic stem cell function when blocking 5E6 F(ab')$_2$ reagents are used. In figure 1 B6 marrow cells were incubated with no NK cells, or NK cells with medium or NK cells with either 5E6 or 4D11F(ab')$_2$ reagents for 24 or 48 hours. CFU-GM assays were performed afterwards. At

the E:T ratio of 1:1, there was no suppression of CFU-GM at 24 hours, but there was suppression at 48 hours. However, there was no difference between normal Ig, 5E6 and/or 4D11 $F(ab')_2$ reagents at this time point. To test effects on myeloid repopulating activity, B6 SCID NK cells were incubated with NMG, 5E6 or 4D11 $F(ab')_2$ for 2 hours, and then were mixed with B6.Ly5.2 marrow cells and cultured for 24 hours. The cells were infused into irradiated B6 (Ly5.1) hosts. The spleen and marrow cells recovered at 7, 14, 21 or 27 days were all of donor origin. There was no effect of the NK cells on CFU-GM in the spleen at any time point. There was a 2-fold defect in CFU-GM in marrow by all groups that had NK cells only on day 14. There were no differences between the 3 different reagents. In figure 3, a similar study was done, except that the incubation time was 48 hours. At day 7, all groups with NK cells had very low yields of CFU-GM, which improved on day 14. However, at this time, the NK cells + 5E6 $F(ab')_2$ group had significantly less CFU-GM in spleen and marrow, compared to all other groups. The other 2 groups involving NK cells had lower yields of CFU-GM than the control marrow group. In Figure 4, a 48 hour incubation was performed. The group with NK cells and 5E6 $F(ab')_2$ was significantly lower in CFU-GM numbers in spleen at day 14 but not 21 or 28, and in marrow at days 14 and 21, but not 28. Therefore, there is some anti-stem cell effect of NK cells plus blocking 5E6 mAb, although long-term reconstitution was not affected. Figure 5, blood neutrophil, RBC and platelet counts were performed at 7, 14, 21, and 28 days. Only at day 14 were there decreased counts of neutrophiles and platelets by all groups with NK cells in the incubation period of 48 hours. Finally, in Figure 6 marrow, NK cells and C1498 leukemia cells were co-incubated in ther presence of 5E6 or 4D11 $F(ab')_2$ reagents. The control group lacked NK cells. The cells were infused into lethally irradiated B6 mice and survival was followed. In addition, aliquots were tested for C1498 tumor colonies in vitro. NK cells decreased C1498 colony formation, but 5E6 was more effective than 4D11. NK cells also enhanced survival $p < 0.001$, and 5E6 was superior to 4D11, $p = 0.0417$. About 50% of the mice in the 5E6 group were cured. This work suggests that this approach has promise for clinical trials with similar reagents.

We have performed lung clearance assays to test the effect of 5E6 $F(ab')_2$ on NK cell mediated function. We have observed that ^{67}Ga.MPO labeled tumor cells can be used for this assay. We can image this isotope with a gamma ray camera, but we hope to extend this to the use of ^{68}Ga.MPO, because this isotope emits protons that is amenable to use with our small animal proton emission tomography (PET) scan apparatus. The data in Figure 5 indicates that this reagent can enhance the ability of NK cells to clear infused tumor cells form the lung. This implies that 5E6 $F(ab')_2$ can act at the very early stage of tumor spread through the blood.

Key research accomplishments

* Blocking negative signals to NK cell Ly49 receptors augments lysis and inhibition of growth of tumor cells in vitro and in vivo
* Blocking negative signals with non-depleting $F(ab')_2$ anti-Ly49I & C mAbs increases survival of mice infused with lethal doses of syngeneic leukemia cells.
* Blocking negative signals to NK cells does have some anti-stem cell activity, a potential pitfall of this approach. However, long-term reconstitution was not affected under the conditions used.
* Imaging assays are being developed to hasten detect tumor at early stages of development and to assess for the effectiveness of newly developed blocking reagents.

Reportable outcomes

* Two manuscripts have been submitted.

1. Koh Crystal Y, Blazar Bruce R, George Thaddeus C, Welniak Lisbeth A, Capitini Christian M, Raziuddin Arati, Murphy William J, and Bennett Michael. 2001 Augmentation of anti-tumor effects by NK cell inhibitory receptor blockade in vitro and in vivo. Blood 97:3132-3137

2.Koh Crystal Y, Raziuddin Arati, Wlniak Lisbeth A, Blazar Bruce R, Bennett Michael, and Murphy William J. 2001 NK inhibitory receptor blockade for purging leukemia: effects on hematopoietic repopulation. Biol Blood Marrow Transplant, in press

* A patent for this approach has been designed
* Funding has been applied for new experiments, based on this work

Conclusions

The ability to block inhibitory signals to NK cells from class I antigens expressed by tumor cells allows more effective anti-leukemia function of NK cells against syngeneic tumors. Moreover, the safety factor of concern, anti-stem cell effects, have been studied. We determined that these latter effects are not sserious. These studies should be extended to the use of a variety of F(ab')2 mAbs to inhibitory receptors. Eventually, we should test this approach with human tumors and blocking antibodies to the human NK cell inhibitory receptors (KIR) by the same type of in vitro methods, and use NOD-SCID mice as hosts of HLA typed tumor cells and HLA type purified CD56+ NK cells.

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Appendices:

Two manuscripts
Five figures

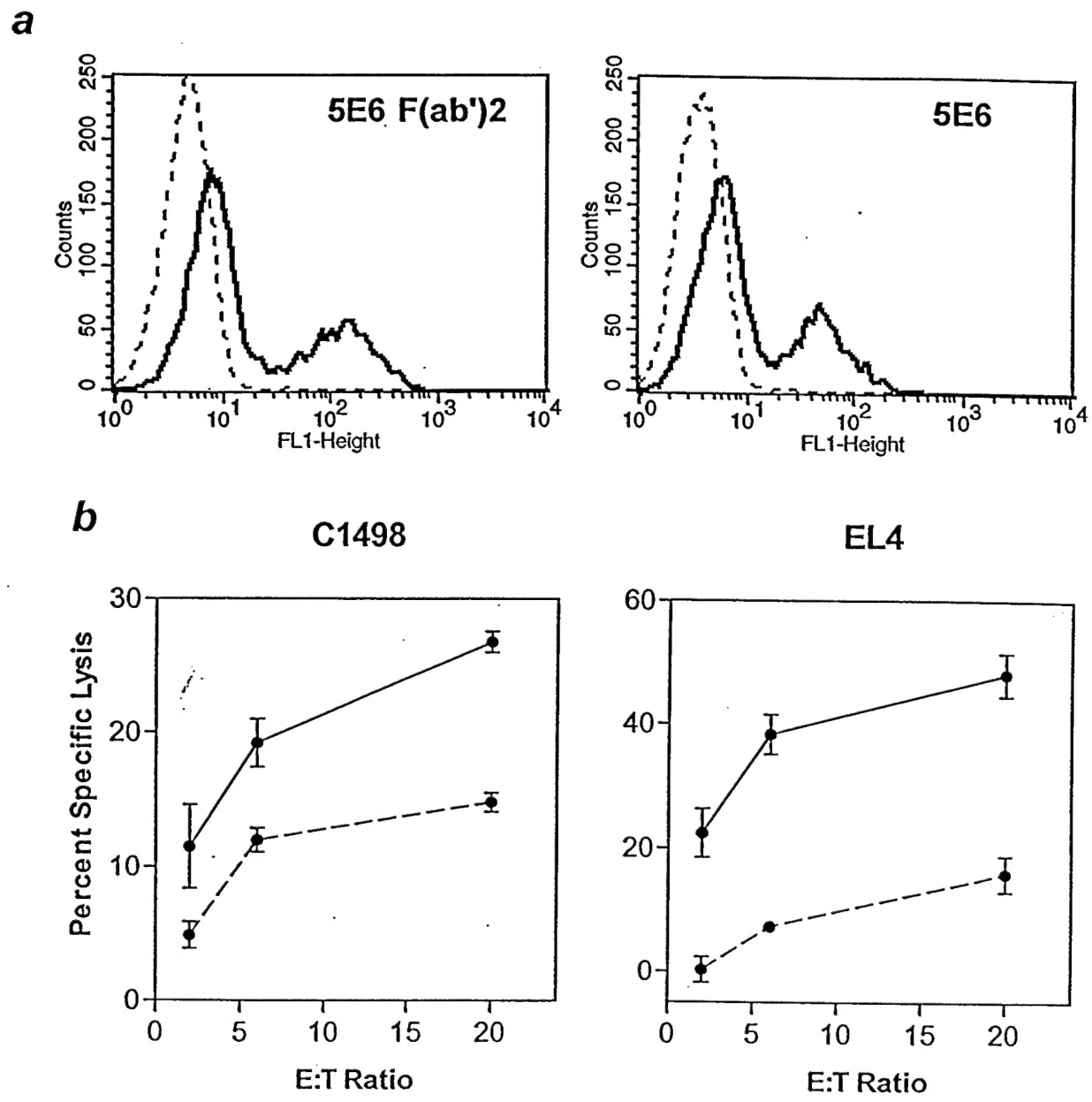
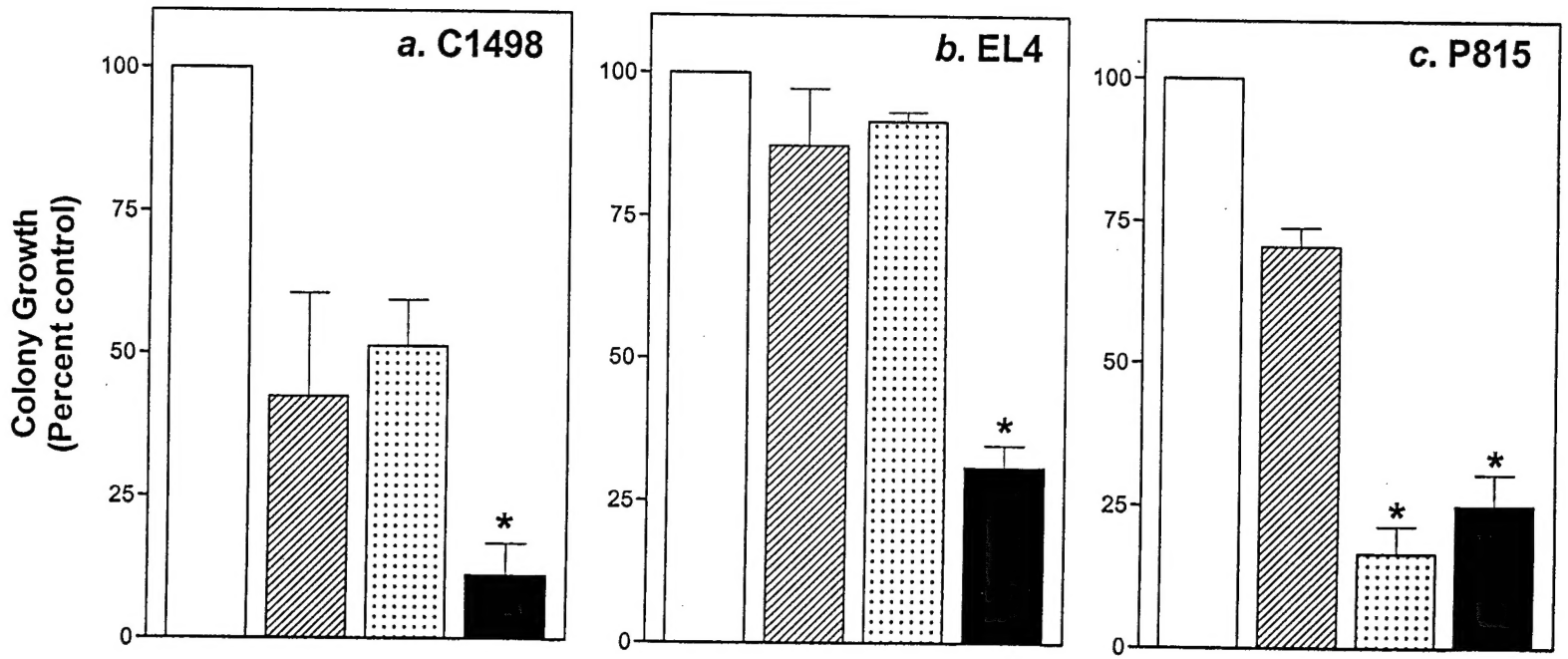


Figure 1. Flow cytometry and cytotoxicity assays of IL-2 activated purified B6 NK cells.
a. Staining with unlabeled F(ab')$_2$ 5E6 mAbs (solid line) or with normal mouse IgG (dashed line), followed by FITC-labeled goat anti-mouse IgG F(ab')$_2$ (left). Staining with biotinylated whole 5E6 mAb (solid line) or not (dashed line), followed by FITC-streptavidin (right). Both reagents stained about 30% of the NK cells. Y-axis, cell number; X-axis, mean fluourescence intensity.
b. Lysis of ^{51}Cr-labeled syngeneic C1498 or EL-4 tumor cells by sorted NK1.1$^+$ 5E6$^+$ NK cells pretreated for 60 min. with normal mouse IgG (dashed lines) or 5E6 F(ab')$_2$ mAb (solid lines) over a 4 hour period. Y-axis, mean and SEM specific lysis, X-axis, effector:target cell rato. At each point, the mean values between control and F(ab')2 were signficantly different ($p < 0.05$).

Figure 2. Treatment of NK cells with 5E6 F(ab')$_2$ results in decreased tumor growth in vitro. Various numbers of B6 SCID NK cells activated with rhIL-2 for 5-7 days were plated in U-bottom 96 well microtiter plates at 50 µl/well and pre-treated with medium alone or 25 µg/ml F(ab')$_2$ fragments of normal mouse IgG (NMG, hatched bars), 5E6 anti-Ly49 I & C (solid bars), or 4D11 anti-Ly49G2 (dotted bars) for 3 h at 37 C. 100, 50 or 25 C1498, EL-4 or P815 cells, respectively, were added at 50 µl/well and the cells were co-cultured for 48 h. As control, tumor cells were cultured alone (open bars). The cells were transferred into a semi-solid matrix and cultured for 5-7 days to detect tumor colonies. C1498 H2^b at NK:tumor ratio of 2:1 (a) EL-4 H2^b at NK:tumor ratio of 10:1 (b) P815 H2^d at NK:tumor ratio 100:1. Data from a representative of 3 experiments are shown, mean % control where control = 100%. The stars indicate significantly lower values than NMG, $p < 0.05$, by Student's t test.



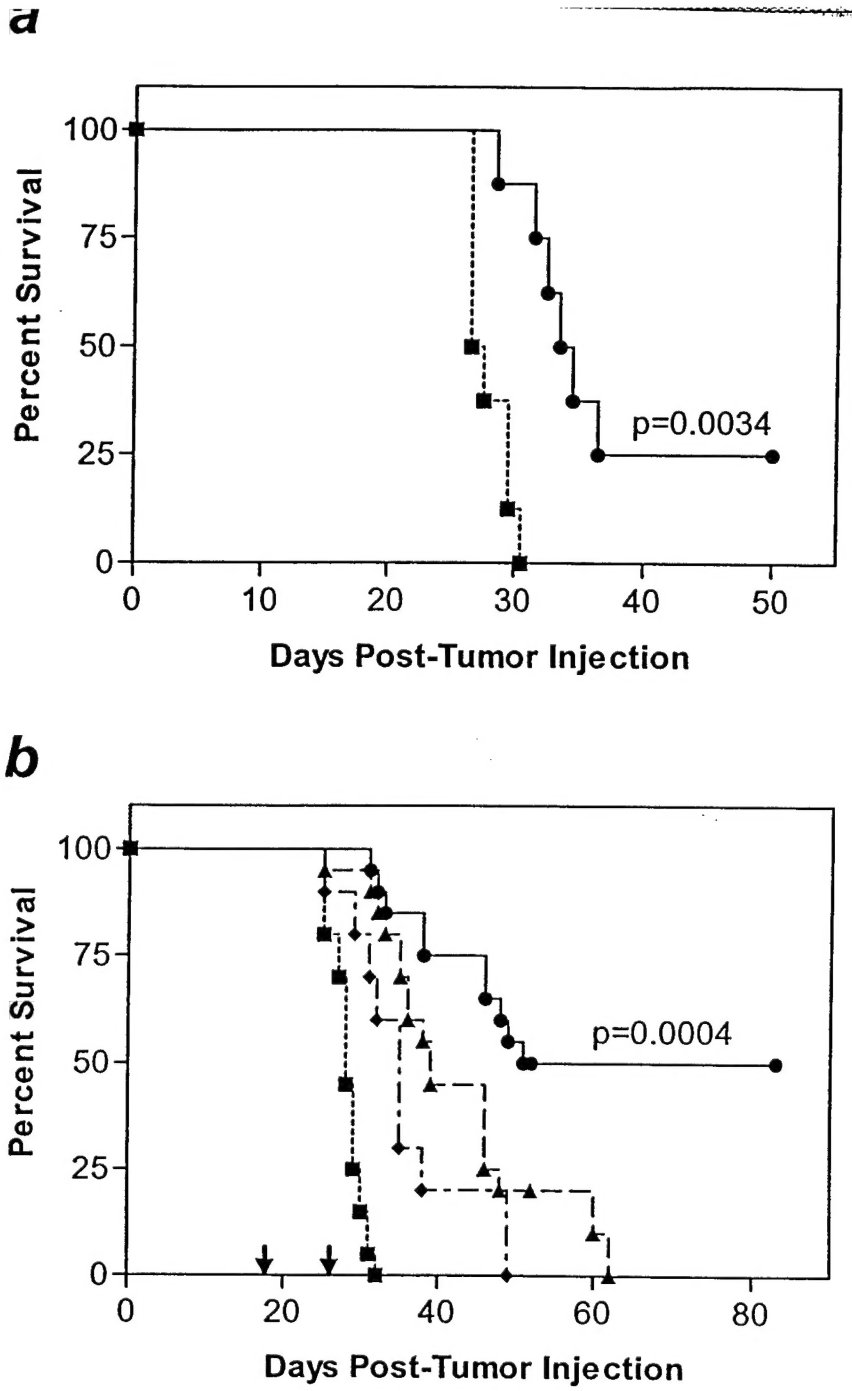


Figure 3. 5E6 F(ab')$_2$ treatment protects B6 mice against C1498 leukemia cells. a. Mice received 180μg 5E6 F(ab')$_2$ (circles) or PBS (squares) i.p. 2 days before and twice weekly for 3 weeks after infusion of 3 X 10^4 C1498 cells. This is a representative of 4 experiments (n = 8 for each group of each experiment. b. 12 x 10^6 IL-2 activated B6 NK cells were incubated with 300 μg 5E6 (circles) or 4D11 (diamonds) F(ab')$_2$ or 5% normal mouse serum (NMS) (triangles) for 2 h at 37 C and further co-cultured with 1.2 X 10^6 C1498 cells for 25 h. As controls, 1.2 X 10^6 C1498 cells were cultured alone (squares). After culture mice received 10^5 C1498 cells and 10^6 NK cells from the cultures i.v. At days 18 and 25 (arrows) mice were infused with inocula of 5 X 10^6 NK cells treated with 5E6 or 4D11 F(ab')$_2$ or NMS ex-vivo followed by IL-2 injection (5 X 10^4 IU i.p.) for 3 days. Pooled data form 2 independent experiments (n = 20/group except for 4D11, n = 10) are shown. The p values were determined by Log-Rank test.

Figure 4. Images of ^{111}In-labeled (220 μCi) YAC-1 lymphoma cells (6 million) infused into a BALB.NK1.1 mouse. A. At 15 min post infusion (27 kcounts from lungs). B.Same mouse 3 hours after infusion (10 kcounts in lung and 16 kcounts in liver/spleen). C. ^{99m}Tc agent was infused just before the 3 hour measurements. It detected counts over the liver from a second channel. D. Overlay of ^{99m}Tc and ^{111}In channels. Initially cells were localized over the lungs, with accummulation of the isotope in the liver and spleen. Post mortem ex vivo determination of ^{111}In showed ratio of lungs to liver of 46%. Thus in vivo imaging provided a closely similar estimate to traditional ex vivo anlysis with potential for long-term dynamic analysis.

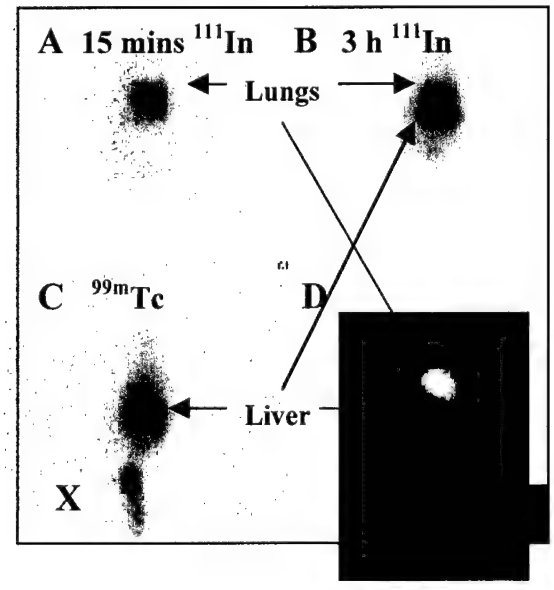
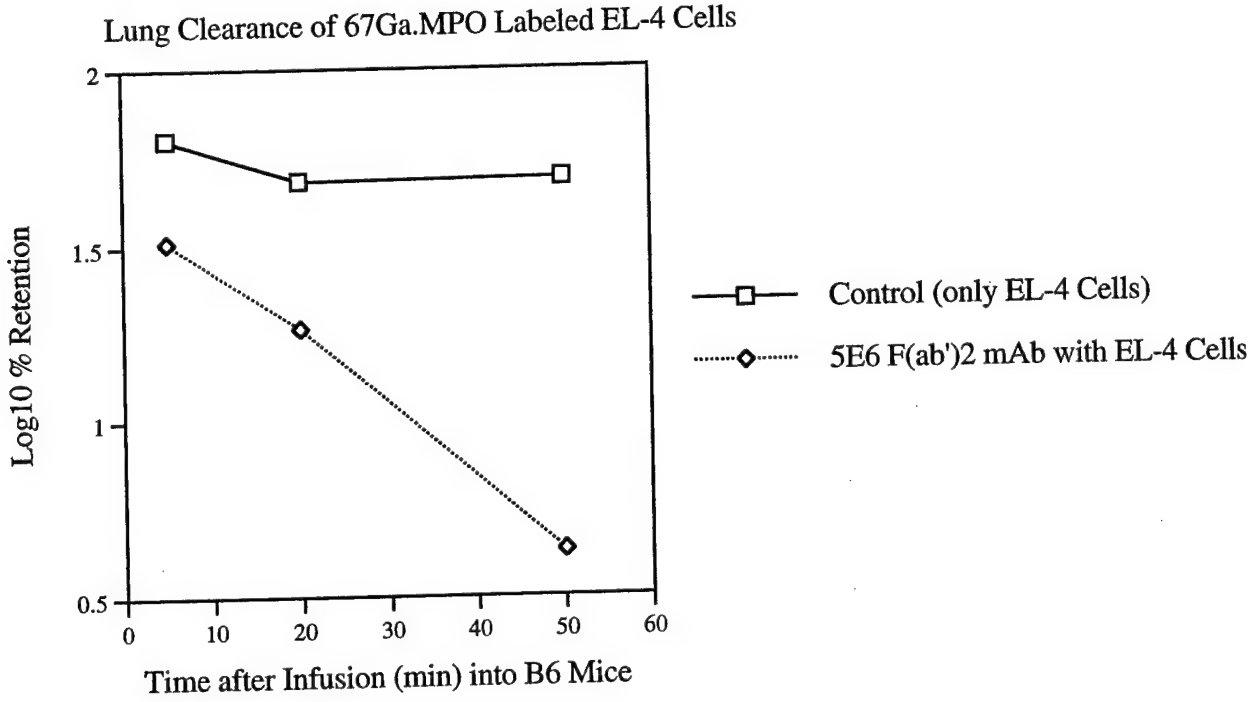


Figure 5. Lung clearance of ^{67}GaMPO labeled syngeneic EL-4 tumor cells by B6 mice treated with 5E6 F(ab')$_2$ mAb. The mice were infkused with 0.5 million EL-4 celsl with or without 5E6 F(ab')$_2$ reagent. B6 mice usually clear El-4 cells rather poorly (see control). The reagent induced very rapid clearance.



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IMMUNOBIOLOGY

Augmentation of antitumor effects by NK cell inhibitory receptor blockade in vitro and in vivo

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Abstract

Subsets of natural killer (NK) cells are characterized by the expression of inhibitory and/or stimulatory receptors specific for major histocompatibility complex (MHC) class I determinants. In mice, these include the Ly49 family of molecules. One mechanism by which tumor cells may evade NK cell killing is by expressing the appropriate MHC class I and binding inhibitory Ly49 receptors. Therefore, the question of whether blocking the interaction between the Ly49 inhibitory receptors on NK and MHC class I cells on tumor cells augments antitumor activity was investigated. Blockade of Ly49C and I inhibitory receptors using $F(ab')_2$ fragments of the 5E6 monoclonal antibody (mAb) resulted in increased cytotoxicity against syngeneic tumors and decreased tumor cell growth in vitro. The effect of 5E6 $F(ab')_2$ was specific for the MHC of the tumor, as the use of $F(ab')_2$ of the mAb against Ly49G2 failed to increase NK activity. Treatment of leukemia-bearing mice with 5E6 $F(ab')_2$ fragments or adoptive transfer of NK cells treated ex vivo with the $F(ab')_2$ resulted in significant increases in survival. These results demonstrate that blockade of NK inhibitory receptors enhances antitumor activity both in vitro and in vivo, suggesting that NK inhibitory receptors can be responsible for diminishing antitumor responses. Therefore, strategies to block inhibitory receptors may be of potential use in increasing the efficacy of immunotherapy. (Blood. 2001;97:3132-3137)

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Introduction

The ability of neoplastic cells to evade the immune system remains a formidable barrier limiting the success of immunotherapy. Tumor cells can employ various mechanisms to escape detection by immune cells. These can include down-regulation of major histocompatibility complex (MHC) class I expression,[1-4] production of immunosuppressive cytokines such as transforming growth factor-β,[1,5] up-regulation of Fas ligand,[6] and deregulation of zeta chain on T cells.[7] In both mouse and human, natural killer (NK) cells are composed of different subsets, which are characterized by the expression of inhibitory and/or activating receptors specific for MHC class I determinants.[8-11] In mice, these receptors belong to the family of Ly49 receptors, which are lectinlike molecules.[12] The human counterpart, killer immunoglobulin-like receptors, belongs to the immunoglobulin superfamily.[11] A small percentage of T cells in mice also express Ly49 receptors.[13] It has been shown that binding of the inhibitory receptors by the appropriate class I molecules results in generation of negative signals leading to inactivation of NK cell functions.[13-15] This inhibitory signal has been shown to dominate over activating stimuli.[15] Furthermore, the rapid rejection of tumors lacking the expression of MHC class I by NK cells demonstrates the pivotal role MHC plays in regulating NK function.[16-18] In spite of these studies demonstrating the functions of the inhibitory receptors in vitro, the in vivo functions of these receptors on either NK or T cells remain to be elucidated.

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One potential means for tumor escape may be by expressing MHC class I determinants at a level that allows sufficient binding of the Ly49 inhibitory receptors and thus escape from NK-mediated killing. In $H2^b$ strains of mice, approximately 35% to 60% of NK cells express inhibitory Ly49C and I receptors that recognize MHC $H2^b$.[19] These NK cells would then be turned off or inactivated by tumors bearing MHC $H2^b$. It has been demonstrated that suppression of inhibitory signals in T cells by blockade of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), results in optimal antitumor effects.[20] Therefore, although NK cells from $H2^b$ strains of mice can kill syngeneic tumors with varying efficiency, greater antitumor activity may be achieved by blocking the interaction between the Ly49 inhibitory receptor family members (ie, Ly49C and I) and their ligand (ie, $H2^b$) present on the tumor.

To examine the effects of blockade of the inhibitory receptors on antitumor activity, we have used a C1498 mouse leukemia model and $F(ab')_2$ fragments of 5E6 monoclonal antibody (mAb),[21] which binds to Ly49C and I receptors, for in vitro as well as in vivo studies. The use of $F(ab')_2$ fragments allowed us to examine the responses that are due to blocking the Ly49 receptors without depletion of the subset in vivo. The results from these studies demonstrate that blockade of Ly49 inhibitory receptors augments NK cell-mediated antitumor effects and that strategies to block NK inhibitory receptor interactions may be of potential use in cancer therapy.

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Mice

C57BL/6 (B6, $H2^b$) mice were obtained from the Animal Production Area (National Cancer Institute at Frederick [NCI-Frederick], MD), and B6 severe combined immunodeficient scid/scid (SCID) mice were generously provided by Dr Robert H. Wiltrout (NCI-Frederick). All mice were kept in a specific pathogen-free condition and used at 8 to 12 weeks of age.

Antibodies and generation of $F(ab')_2$ fragments

Antimouse FcγR (2.4G2, rat immunoglobulin [Ig]-G2a), fluorescein isothiocyanate (FITC)-conjugated anti-NK1.1 (PK136, mouse IgG2a), and biotinylated anti-Ly49C and I (5E6, mouse IgG2a) were purchased from Pharmingen (San Diego, CA). Phycoerythrin (PE)-conjugated streptavidin and $F(ab')_2$ fragments of normal mouse IgG (NMG) were purchased from Jackson ImmunoResearch (West Grove, PA). $F(ab')_2$ fragments of anti-Ly49C and I (5E6) and anti-Ly49G2 (4D11, rat IgG2a; the hybridoma was a gift from Dr John Ortaldo, NCI-FCRDC) mAbs were prepared as previously described.[21] Briefly, the antibodies were purified from ascites fluid by affinity column purification, concentrated to 5 mg/mL, digested with pepsin, and neutralized with 2 M Tris base solution. The neutralized digestion mixture was dialyzed against phosphate-buffered saline (PBS) overnight. The efficiency of digestion and purity of the resulting $F(ab')_2$ fragments were checked by 4%- to 20%-gradient sodium dodecyl sulfate- polyacrylamide gel electrophoresis.[21]

Cell lines

All cell lines were obtained from American Type Culture Collection (Rockville, MD). C1498 ($H2^b$) is a murine leukemia, and EL4 ($H2^b$) is a murine T-cell lymphoma. P815 ($H2^d$) is a murine mastocytoma. Frozen stocks of the cell lines were thawed every 2 months for in vitro assays.

SCID NK cell culture and cell sorting

Single-cell suspensions of splenocytes and bone marrow cells from B6 SCID mice were prepared with red cell lysis. Cells were cultured in NK cell media (RPMI 1640 supplemented with 10% fetal bovine serum [FBS], 100 U/mL penicillin/streptomycin, 2 ? 10^3 μM L-glutamine, 10 mM Hepes, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 2.5 ? 10^{-5} M 2-ME, and 1 μg/mL indomethacin) containing 5000 IU/mL recombinant human interleukin-2 (rhIL-2) (Biological Response Modifiers Program, NCI-FCRDC) at 0.5 to 1 ? 10^6 cells per milliliter for 5 to 7 days. For cytotoxicity assays, a pure population of $NK1.1^+5E6^+$ and $NK1.1^+5E6^-$ NK cell subsets were sorted from B6 SCID splenocytes and cultured in NK media containing 5000 IU/mL rhIL-2 for 5 to 7 days as previously described.[21]

Cytotoxicity assay

We incubated 1.5 to 2 ? 10^6 C1498 or EL4 targets with 150 to 250 μCi sodium chromate (^{51}Cr) (Amersham Life Science, Arlington Heights, IL) for 1.5 hours at 37°C. Radiolabeled cells were washed and diluted to an appropriate concentration. Various numbers of $NK1.1^+5E6^+$ or $NK1.1^+5E6^-$ effectors were added first to the wells of V-bottom 96-well microtiter plates in triplicates. For assays determining the effect of 5E6 $F(ab')_2$, the effectors were preincubated with 40 to 60 μg/mL 5E6 $F(ab')_2$ for 30 to 60 minutes at 37°C and

then cocultured with the targets for 4 hours. ^{51}Cr activity in the culture supernatant was then measured in a liquid scintillation counter, and the percentage of specific lysis was calculated as previously described.[21]

Clonogenic assays

Various numbers of B6 SCID NK cells activated with rhIL-2 for 5 to 7 days were plated in U-bottom 96-well microtiter plates to achieve NK-to-tumor ratios of 100:1, 50:1, 10:1, 5:1, 2:1, or 1:1 and pretreated with media alone or 25 μg/mL $F(ab')_2$ fragments of NMG, 5E6, or 4D11 for 2 to 3 hours at 37°C in complete Iscoves modified Dulbecco medium (IMDM) (supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 2 ? 10^3 μM L-glutamine, 5 ? 10^{-5}M 2-ME, and 5000 IU/mL IL-2). Then 100, 50, or 25 C1498, EL4, or P815 cells, respectively, were added per well, and the cells were cocultured for 48 hours. The cocultured cells were collected and transferred into the colony assay media (prepared the same as complete IMDM except for 1.1% methyl cellulose [wt/vol] and no rhIL-2) and plated in 35-mm petri dishes in triplicates. Cultures were incubated for 6 to 7 days in humidified atmosphere at 37°C with 5% CO_2. Colonies were enumerated on a stereo microscope (Nikon, Melville, NY).

Survival studies

C1498 leukemia cells were freshly thawed 7 days prior to in vivo administration and kept in log phase until use. In some experiments, B6 mice were treated with either 5% normal mouse serum (NMS) or 180 μg 5E6 $F(ab')_2$ per mouse (intraperitoneally [ip]) 2 days prior to the injection of tumor cells. At day 0, mice were injected (intravenously [iv]) with a lethal dose of C1498 (1 ? 10^5 cells), and beginning on the day of tumor injection, mice were treated with 5E6 $F(ab')_2$ twice a week for 3 to 4 weeks. In the experiments indicated, 10 to 12 ? 10^6 IL-2-activated NK cells were preincubated with 300 μg 5E6 or 4D11 $F(ab')_2$ or 5% NMS for 2 hours at 37°C and further cocultured with 1 to 1.2 ? 10^6 C1498 leukemia cells in 5 mL total volume in 6-well culture plates for 24 hours. As controls, 1 to 1.2 ? 10^6 C1498 cells were cultured alone. After the coculture, mice were injected with the cocultured cells at 1 ? 10^5 C1498 cells and 1 ? 10^6 NK cells per mouse (iv), a dose based on the cell concentration at the initiation of the cocultures. In some experiments, adoptive transfer of NK cells was performed in mice that were injected with the cocultured NK and tumor cell mixtures described above. At days 18 and 25 post-initial NK plus tumor cell injection, 6 ? 10^7 IL-2-activated NK cells were preincubated with 500 μg 5E6 or 4D11 $F(ab')_2$ or 5% NMS for 2 hours at 37°C, and without washing, the cells were diluted in PBS at 1 ? 10^7/mL. The ex vivo-treated NK cells were then transferred into tumor-bearing mice at 5 ? 10^6 per mouse (iv), and all mice were treated with 5 ? 10^4 IU rhIL-2 (ip) for 3 consecutive days beginning on the day of adoptive transfer. For all in vivo studies, 6 to 8 mice per group were used in each experiment, and 2 to 3 independent experiments were performed for the various conditions.

Statistics

The statistical analyses were performed by means of the Student *t* test to determine the significance of the differences between experimental and control groups for in vitro assays. The *P* values for the survival studies were obtained with the log-rank test.

Results

Stable binding of 5E6 $F(ab')_2$ to Ly49C and I receptors enhances NK cell cytotoxicity

To assess the effects of blocking NK cell inhibitory receptors with $F(ab')_2$ fragments of 5E6 mAb, we first examined whether stable binding of 5E6 $F(ab')_2$ fragments on IL-2-activated NK cells can be maintained. We used SCID mice as a source of NK cells since they lack T and B cells and since culture of SCID splenocytes in IL-2 gives rise to a relatively pure population of NK cells.[1] As shown in Figure 1A, binding of 5E6 $F(ab')_2$ could be detected at 37°C, and the level of expression was comparable to $5E6^+$ subset detected by whole antibody. In addition, binding of the $F(ab')_2$ fragments to Ly49C and I receptors was maintained over a 24- to 48-hour culture period (data not shown). We then determined if blocking the interaction of MHC class I expressed on C1498 ($H2^b$) leukemia or EL4 ($H2^b$) lymphoma cell lines and Ly49C and I receptors on NK cell subsets can result in greater cytotoxicity. In these studies, the $5E6^+$ and $5E6^-$ subsets were sorted from B6 ($H2^b$) SCID splenocytes. The sorted cells were activated with rhIL-2 for 5 to 7 days and used as effectors against C1498 or EL4 cells in a standard 4-hour cytotoxicity assay. The results show that C1498 and EL4 cells could be killed by the $5E6^-$ subset at various levels at an effector-to-target ratio of 20:1 (18.4% ± 1.2% and 49.8% ± 1.8% specific lysis for C1498 and EL4, respectively). In contrast, both tumor cells were relatively resistant to cytotoxicity mediated by the $5E6^+$ NK subset (Figure 1B), and when $5E6^+$ NK cells were pretreated with 5E6 $F(ab')_2$, cytotoxicity against both targets was significantly increased (Figure 1B) to levels comparable to or higher than that mediated by

the $5E6^-$ subset. However, pretreatment of the $5E6^+$ subset with the $F(ab')_2$ fragment did not affect the level of cytotoxicity against an NK-sensitive target, YAC-1 [70.8% ± 1.7% vs 75.1% ± 6.5% specific lysis with vs without 5E6 $F(ab')_2$]. These results demonstrate that negative signals induced via the inhibitory receptors suppress the killing ability of NK cell subsets and that blocking these inhibitory signals augments cytotoxicity of this subset.

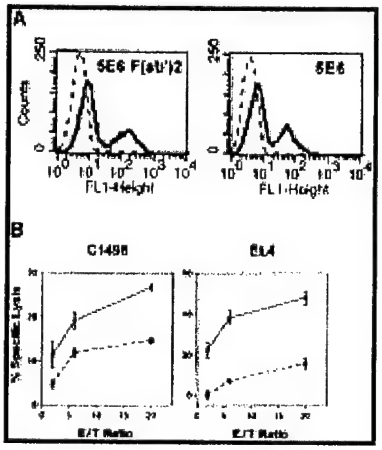

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Figure 1. Effect of 5E6 $F(ab')_2$ fragments on $5E6^+$ NK cells. The 5E6 $F(ab')_2$ fragments bind $5E6^+$ NK cells and increase cytotoxicity mediated by the $5E6^+$ NK cell subset. (A) IL-2-activated NK cells (5 ? 10^6) were incubated with $F(ab')_2$ fragments of 5E6 (solid histogram) at 1 ? 10^6/mL or with normal mouse IgG (dashed histogram) at 25 μg/mL for 2 hours at 37°C and stained with FITC goat antimouse IgG specific for $F(ab')_2$ fragments (left panel). As controls, NK cells were incubated alone and stained with biotinylated 5E6 and secondary antibody, FITC-streptavidin (right panel, solid histogram) or the secondary antibody only (dashed histogram). (B) The $NK1.1^+5E6^+$ subset was sorted from B6 SCID splenocytes by flow cytometry, activated with rhIL-2, and used at day 5 as effectors against C1498 or EL4 targets in standard 4-hour cytotoxicity assay as described previously.[21] Various numbers of $NK1.1^+5E6^+$ effectors were pretreated with media (■) or 5E6 $F(ab')_2$ (●), and the effectors were then cocultured with the targets for 4 hours, after which ^{51}Cr activity was measured in a liquid scintillation counter. A representative of 3 independent experiments is shown.

Blockade of Ly49C and I receptors inhibits tumor growth in vitro

To determine whether blockade of Ly49C and I receptors increases the NK-mediated inhibition of tumor growth in vitro, IL-2-activated NK cells from B6 SCID mice were preincubated with $F(ab')_2$ fragments of either 5E6 or 4D11, a mAb against Ly49G2 specific for $H2D^d$ used as a control for specificity, for 2 to 3 hours. Only the 5E6 $F(ab')_2$ should have an effect on NK cells with tumors expressing $H2^b$. NK cells were then incubated with syngeneic tumors, C1498 or EL4 (both $H2^b$), or an allogeneic tumor, P815 ($H2^d$), all of which express neither Ly49C and I nor Ly49G2 (data not shown), at different NK-to-tumor ratios for 48 hours. After coculture with NK cells, viable tumor cell counts were assessed in clonogenic assays. As shown in Figure 2A, clonogenic growth of C1498 leukemia cells was inhibited by co-incubation with NK cells compared with the growth of untreated cells, and this growth inhibition was further increased when 5E6 $F(ab')_2$ was added ($P < .05$). Although the growth of EL4 was not affected by NK cells, pre-incubation with 5E6 $F(ab')_2$ also significantly decreased the growth of EL4 ($P = .00076$; Figure 2B). In contrast to the effects observed with 5E6 $F(ab')_2$, growth of neither C1498 nor EL4 was affected by treatment of NK cells with 4D11 $F(ab')_2$ (Figure 2A,B). However, the growth of P815 cells, which were relatively resistant to even allogeneic NK cells, was significantly decreased in the presence of 4D11 $F(ab')_2$ ($P < .01$; Figure 2C). Interestingly, the 5E6 $F(ab')_2$ also exerted an effect on growth of P815 ($P < .01$), corroborating previous reports showing that Ly49C and I inhibitory receptors are capable of binding, albeit weakly, to $H2^d$ in vitro.[22] The addition of 5E6 or 4D11 $F(ab')_2$ alone to the tumor cells did not have a significant effect on their growth (data not shown).

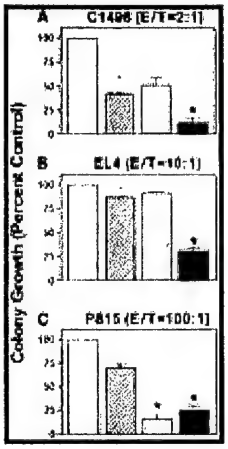

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Figure 2. Treatment of NK cells with 5E6 F(ab')$_2$. Treatment of NK cells with 5E6 F(ab')$_2$ results in decreased tumor growth in vitro. Various numbers of B6 SCID NK cells activated with rhIL-2 for 5 to 7 days were plated in U-bottom 96-well microtiter plates at 50 μL per well and pretreated with media alone (☐) or 25 μg/mL F(ab')$_2$ fragments of normal mouse IgG (NMG) (▨), 5E6 (Ly49C and I) (■), or 4D11 (Ly49G2) (▩) for 2 to 3 hours at 37°C. Then 100, 50, or 25 C1498, EL4, or P815 cells, respectively, were added at 50 μL per well, and the cells were cocultured for 48 hours. As controls, tumor cells were cultured alone. After the co-incubation, the cells were transferred into a semisolid matrix and cultured for 5 to 7 days for quantification of leukemic cell colonies. (A) C1498 ($H2^b$) cells at NK-to-tumor ratio of 2:1. (B) EL4 ($H2^b$) at NK-to-tumor ratio of 10:1. (C) P815 ($H2^d$) at NK-to-tumor ratio of 100:1. Data from a representative of 3 independent experiments are shown as a percentage of control cells, where tumor growth in the absence of NK and the antibody is used as 100%. The stars indicate significant differences in 5E6 or 4D11 F(ab')$_2$-treated groups compared with NMG controls as determined by Student *t* test.

Treatment of leukemia-bearing mice with 5E6 F(ab')$_2$ or adoptive transfer of NK cells treated ex vivo with F(ab')$_2$ increases the survival rate

To address whether inhibitory receptor blockade can also result in greater antitumor activity in vivo, the C1498 acute leukemia model was employed. B6 mice were treated with 5E6 F(ab')$_2$ or NMS 2 days prior to injection of a lethal dose of C1498. The animals were further treated with the antibody or the control twice a week for 3 weeks post-tumor injection and monitored for survival. As shown in Figure 3A, injection of C1498 leukemia cells resulted in death of 87% of the controls in which extensive tumor growth was observed in the liver, bone marrow, and central nervous system. Treatment of mice with the 5E6 F(ab')$_2$ resulted in a significant increase in survival (P = .021), with 50% of mice remaining disease-free up to 100 days post-tumor injection. In contrast, treatment of mice with 5E6 whole antibody to deplete the $5E6^+$ NK cell subset did not have any effect on the survival of mice compared with the NMS control even though $5E6^+$ cells decreased from 36.2% to 7.3% of NK cells after the treatment (data not shown). No toxicities, effects on hematologic parameters, or changes in the pattern of tumor growth were observed in mice receiving 5E6 F(ab')$_2$ (data not shown).

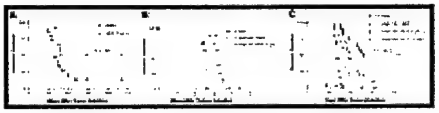

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Figure 3. Effect of 5E6 F(ab')$_2$ on mice injected with a lethal dose of C1498. 5E6 F(ab')$_2$ treatment of mice injected with a lethal dose of C1498 results in increased survival rate. (A) B6 mice were treated with 180 μg 5E6 F(ab')$_2$ or 5% NMS (ip) 2 days prior to injection with 1 ? 10^5 C1498 cells (iv). The animals were then treated with 180 μg 5E6 F(ab')$_2$ per dose or NMS twice a week for 3 weeks and monitored for survival. Pooled data from 2 independent experiments (n = 15 and 12 for NMS and 5E6 F(ab')$_2$ groups, respectively) is shown. (B) IL-2-activated NK cells (12 ? 10^6) were preincubated with 300 μg 5E6 F(ab')$_2$ or 5% NMS for 2 hours at 37°C and were further cocultured with 1.2 ? 10^6 C1498 leukemia cells for 24 hours. As controls, 1.2 ? 10^6 C1498 cells were cultured alone. After the coculture, cells were washed and mice were injected with 1 ? 10^5 C1498 and 1 ? 10^6 NK cells per mouse (iv), a dose based on the cell concentration at the initiation of coculture (n = 8 per group). A representative of 3 independent experiments is shown. (C) B6 mice were injected with cocultured NK and C1498 cell mixture as in panel B. In addition, a group of mice were injected with NK and C1498 cell mixture that was cocultured in the presence of 300 μg 4D11 F(ab')$_2$. At days 18 and 25 post-initial tumor plus NK cell injection (indicated by arrows), mice in the appropriate group were injected with NK cells treated with NMS or 5E6 or 4D11 F(ab')$_2$ (5 ? 10^6 per mouse, iv) followed by IL-2 injection (5 ? 10^4 IU per mouse, ip) of all mice for 3 consecutive days. Pooled data from 2 independent experiments (n = 20 per group except for 4D11 F(ab')$_2$-treated group in which n = 10) are shown. The P value indicates a significant difference between groups injected with NK cells treated with 5E6 or 4D11 F(ab')$_2$ as determined by log-rank test.

While in vivo administration of 5E6 $F(ab')_2$ demonstrates that blockade of Ly49C and I inhibitory receptors increases antitumor activity, it does not distinguish NK-specific activity from that of the minor population of T cells also expressing the inhibitory receptors.[13] To rule out the involvement of this T-cell subset, mice were injected with the mixture of C1498 and NK cells that were cocultured with or without 5E6 $F(ab')_2$ for 24 hours. Reduction in tumor growth that results from the coculture of NK and tumor cells in the presence of 5E6 $F(ab')_2$ was not sufficient to affect survival compared with the control group that received NK and C1498 cocultures without 5E6 $F(ab')_2$ (Figure 3B). Our preliminary observation on the kinetics of C1498 growth in vivo suggested that the leukemia cells appear to remain dormant for 2 to 3 weeks, after which rapid tumor growth is detected in the animal (data not shown). Therefore, we examined whether adoptive transfer of NK cells preincubated ex vivo with the $F(ab')_2$ into the tumor-bearing mice can result in increased antitumor effects. Adoptive transfer of NK cells that were treated with NMS, 5E6, or 4D11 $F(ab')_2$ was performed at days 18 and 25 after the initial injection of the animals with the tumor and NK cell mixture that was cocultured for 24 hours. Figure 3C demonstrates that adoptive transfer of IL-2-activated NK cells treated with 5E6 $F(ab')_2$ ex vivo resulted in a significant increase in the survival of mice, with 50% long-term survivors (P = .0004). In contrast, no significant difference in survival was observed between mice injected with NK cells treated with NMS or 4D11 $F(ab')_2$. Taken together, these results demonstrate that immunotherapy using NK cells with blockade of Ly49C and I receptors using 5E6 $F(ab')_2$ fragments augments antitumor effects in vivo.

Discussion

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The concept of removing inhibitory signals to optimize immune responses has been receiving increased attention with regard to cancer therapy. It has been demonstrated that CTLA-4 blockade results in enhancement of antitumor immunity against poorly immunogenic tumor cells and increased memory responses.[20] Similarly, blocking NK inhibitory receptors may be necessary for optimal antitumor effects mediated by NK cells, even those activated with stimulatory cytokines, such as IL-2. In this study, we have shown that blockade of NK inhibitory receptors can promote antitumor responses both in vitro and in vivo.

Previous studies have demonstrated that modulation of MHC class I expression on various tumor cells influences the susceptibility of these cells to NK-mediated cytotoxicity.[23] The inability of the $5E6^+$ NK cell subset to effectively lyse C1498 or EL4 tumor cells bearing $H2^b$ demonstrates that class I expression by tumor cells can play an important role in evasion from detection by NK cell subsets. In spite of comparable levels of class I expression between C1498 and EL4 cells, the extent of resistance to NK killing exhibited by C1498 is different from EL4 (Figure 1B). NK cells may recognize other tumor-specific determinants via activating receptors[9,24,25] or other receptors that contribute to tumor cells' susceptibility to lysis. However, the increase in cytotoxicity that is mediated by the $5E6^+$ subset as a result of the blockade of Ly49C and I receptors indicates that inhibition of the negative signals induced via the inhibitory receptors enhances antitumor effects.

Whereas the 4-hour cytotoxicity assays show the effects of 5E6 $F(ab')_2$ on the sorted NK cell subset, the same effect could not be observed if a mixed population of NK cells was used (data not shown). This may be due to the sensitivity of a short-term cytotoxicity assay to detect the differences in the subset of NK cells. However, we have demonstrated that blockade of Ly49C and I receptors resulted in a significant inhibition of tumor growth in vitro even if a mixed population of NK cells was used in clonogenic assays. More importantly, the specificity of the suppressive effects on tumor growth was demonstrated with the use of $F(ab')_2$ fragments of both 5E6 and 4D11. Blockade of Ly49C and I receptors, which have been shown to bind $H2^b$ as well as $H2^d$,[22] decreased the growth of tumors bearing MHC class I of either haplotype. However, blockade of Ly49G2 resulted in decreased growth of P815 ($H2^d$) only, demonstrating the specificity of the inhibitory receptor blockade for the Ly49 determinants binding the appropriate MHC.

Recently, it has been reported that activation and recruitment of NK cells to the sites of tumor growth depend on the expression of MHC class I by the tumor.[26] However, the relationship between MHC class I expressed by the tumor and particular NK subsets was not assessed. In this study, increased survival of tumor-bearing mice treated with 5E6 $F(ab')_2$ (Figure 3A) demonstrates that this subset is responsible for decreased antitumor activity against the syngeneic tumor and that blockade of Ly49C and I receptors increases antileukemia effects. We have observed that culturing C1498 with NK cells in the presence of 5E6 $F(ab')_2$ prior to injection does not have a significant effect on the survival of mice compared with the control cocultures without the $F(ab')_2$ fragments (Figure 3B). Our data also show that adoptive transfer of 5E6 $F(ab')_2$-treated NK cells is more effective in promoting antitumor effects compared with control-treated NK cells when mice were challenged with a reduced lethal dose of tumor cells by prior exposure to NK cells; however, when mice were injected with a higher dose,

increased antitumor effects mediated by 5E6 $F(ab')_2$-treated NK cells could not be observed (data not shown). These results suggest that there may be a threshold at which the animals succumb to tumor. While tumor growth measured in vitro is significantly reduced by blockade of Ly49C and I during the coculture (Figure 2), the coculture alone may not be sufficient to reduce the tumor burden below the threshold level in vivo. However, in vivo studies indicate that continual blockade of NK inhibitory receptors by 5E6 $F(ab')_2$ treatment of animals or multiple injections of NK cells that are treated ex vivo with the antibody augments antitumor effects and can reduce the tumor burden below the threshold level.

As certain subsets of NK cells express inhibitory receptors that recognize self-MHC, it is possible that autoreactivity may result from the blockade of these inhibitory receptors. In vitro studies using cocultures of activated NK and bone marrow cells with or without $F(ab')_2$ fragments of the mAbs as well as in vivo studies, such as bone marrow transplantation with NK cells treated with $F(ab')_2$ fragments of mAbs against Ly49 inhibitory receptors, indicated no adverse effects of Ly49C and I blockade on growth of normal hematopoietic progenitors (manuscript in preparation).

It has been suggested that NK cells are responsible for controlling a low tumor burden at an initial stage until the adaptive arm of the immune system plays a major role in mediating antitumor responses.[1,27] Therefore, blocking the inhibitory receptors expressed on a major subset of NK cells can be a powerful means to eradicate tumors when the tumor burden is minimal as that occurs after cytoreductive therapy. The significant effects observed with blockade of a subset representing 35% to 60% of NK cells also suggest that the combination of antibodies against multiple subsets may lead to even more potent antitumor responses. The effectiveness of a blockade of Ly49 inhibitory receptors in a purging model, in which animals are transplanted with bone marrow cells contaminated with tumors and $F(ab')_2$-treated NK cells, is currently being investigated. The results from our studies also suggest that the NK inhibitory receptor blockade may be applied to enhance immune responses to viral infections, such as murine cytomegalovirus, which encodes an MHC class I homologue to evade the immune system.[28,29]

Taken together, these results demonstrate for the first time that Ly49 receptors expressed on NK cell subsets can be responsible for diminishing antitumor responses and that blockade of these receptors augments antitumor effects. Thus, blockade of NK inhibitory receptors can be of potential use in increasing the efficacy of immunotherapy for cancer.

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Footnotes

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NK inhibitory receptor blockade for purging of leukemia: effects on hematopoietic reconstitution

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Short Title: Effects of NK inhibitory receptor blockade on hematopoietic cells

ABSTRACT

One of the obstacles of bone marrow transplantation (BMT) that limit its efficacy is failure to eradicate the original tumor. The incidence of tumor relapse is particularly high after autologous BMT. Natural killer (NK) cells are comprised of various subsets that can express inhibitory receptors for MHC class I determinants. We have recently demonstrated that blockade of NK cell inhibitory receptors can augment anti-tumor effects in vitro and in vivo. However, breakdown of tolerance and autoreactivity may occur as a result of inhibition of NK cell inactivation to self MHC determinants. We have utilized $F(ab')_2$ fragments of monoclonal antibody, 5E6, against Ly49C and I inhibitory receptors, which are expressed on 35-60% of NK cells in $H2^b$ strains of mice and are specific for $H2K^b$, to investigate the effect of inhibitory receptor blockade on syngeneic bone marrow cell (BMC) and tumor cell growth. We show that treatment of IL-2 activated C57BL/6 (B6, $H2^b$) SCID mouse NK cells with 5E6 $F(ab')_2$ during 48-hour co-culture resulted in autoreactivity against syngeneic BMC as demonstrated by suppression of myeloid reconstitution at day 14 post-BMT. However, this suppressive effect was transient and normalized by day 21 post-BMT. In contrast, blockade of inhibitory receptors during 24-hour co-culture had no adverse effects on myeloid reconstitution after BMT. Furthermore, under the same co-culture conditions, NK cell-mediated purging of C1498 leukemia cells contaminating syngeneic BMC was more effective with inhibitory receptor blockade leading to a significantly higher proportion of animals with long-term survival compared to the control recipients. These results demonstrate that short-term in vitro blockade of inhibitory receptors can augment anti-tumor activity without long-term inhibitory effects on BMC and thus may be of potential use in purging of contaminating tumor cells prior to autologous BMT.

INTRODUCTION

Bone marrow transplantation (BMT) and peripheral blood stem cell (PBSC) transplantation are currently used as a treatment for a variety of malignant and non-malignant diseases [1]. However, effective is limited due to serious and often fatal complications associated with BMT or PBSC transplantation. In particular, autologous BMT and PBSC transplantation are associated with a high rate of tumor relapse in part due to the original tumor cells contaminating the bone marrow or peripheral blood and lack of a graft-vs.-tumor effect [2,3]. To overcome this obstacle, different physical, clinical, or immunological means have been used to purge the contaminating tumor cells ex vivo [4-6] including the use of activated autologous NK cells [7,8]. NK cells, initially described as cells with the ability to kill some tumor cells in a non-MHC restricted manner, have been shown to employ various cytotoxic mechanisms such as perforin/granzyme- or FasL-mediated pathway [9,10]. In addition, activated NK cells produce cytokines such as IFN-γ and TNF-α, which mediate anti-tumor effects [11-13].

NK cells are composed of subsets that are characterized by the expression of various inhibitory and/or activating receptors. In mice, these receptors belong to two families of C-type lectins, Ly49 receptor family specific for MHC class I or CD94/NKG2 receptor family specific for non-classical class I, Qa-1 [14,15]. Similarly, human NK cell subsets express killer cell immuoglobulin-like receptors (KIR) that are inhibitory and/or activating receptors belonging to immunoglobulin super family [16] or C-type lectin, CD94/NKG2 [17,18] inhibitory receptor family. Similar to the mouse counterpart, these receptors are specific for HLA class I molecules [19-21]. Inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domains [14,16]. Upon binding of MHC class I by the inhibitory receptors,

phosphorylation of the tyrosine residue in ITIM occurs leading to transduction of inhibitory signals downstream and thus inactivation of NK cells [14,16]. On the other hand, activating receptors lack ITIM in their cytoplasmic domain; instead, they are associated with an adapter protein, such as DAP-10 or DAP-12, which contains in its cytoplasmic domain the immunoreceptor tyrosine-based activating motif (ITAM) [22,23]. Crosslinking of activating receptors by their MHC class I ligands results in increased cytotoxicity and cytokine production by the NK cell subset expressing the receptor [24,25]. However, it has been shown that inhibitory signals, which are dominant over activating signals, usually dictate the functional outcome of NK cell subsets [26,27]

Therefore, tumor cells can evade NK cell-mediated killing by regulating the level of MHC class I expression at which they can interact with the inhibitory receptors leading to inactivation of NK cells. For example, in $H2^b$ strains of mice, 35-60% of NK cells express Ly49C and I receptors specific for $H2K^b$ molecules [28], and tumor cells bearing $H2^b$ can bind to Ly49C and I inhibitory receptors and inactivate the subset, which represents a significant portion of NK cells. We have recently demonstrated that blockade of Ly49C and I inhibitory receptors using $F(ab')_2$ fragments of antibody against the inhibitory receptors (5E6) augments anti-tumor activity mediated by NK cells both in vitro and in vivo [29]. Therefore, blocking the interaction between inhibitory receptors and MHC class I on NK and tumor cells, respectively, during the purging procedure may enhance the anti-tumor effects by blocking NK cell subset inactivation upon binding MHC -bearing tumor cells. However, this inhibition of NK cell inactivation by blocking receptors specific for "self" MHC may also result in autoreactivity against hematopoietic stem cells or progenitors resulting in failure of lymphohematopoietic reconstitution. Therefore, it was of importance to examine if NK inhibitory receptor blockade

exert suppressive effects on the hematopoietic progenitors and to determine the purging condition, in which inhibitory receptor blockade can be utilized to optimize NK cell-mediated anti-tumor effects without affecting hematopoietic cells.

To address this, we have employed a co-culture system and a syngeneic BMT model. Lethally irradiated B6 ($H2^b$) mice were transplanted with B6 SCID NK cells and B6 BMC or B6 SCID NK cells and B6 BMC contaminated with syngeneic tumor cells that were co-cultured with or without $F(ab')_2$ fragments of anti-Ly49C and I mAb (5E6). $F(ab')_2$ fragments were used to ensure blockade of the inhibitory receptors without activation via FcγR in vitro during the co-culture and depletion of the antibody binding subset in vivo. In this report, we present data, which demonstrate that long-term blockade of NK inhibitory receptors results in transient suppression of myeloid reconstitution after syngeneic BMT. On the other hand, short-term blockade of NK inhibitory receptors during purging of tumor cells can augment NK cell-mediated anti-tumor activity without adverse effects on hematopoietic reconstitution.

MATERIAS AND METHODS

Mice. C57BL/6 (B6, $H2^b$) and B6.Ly5.2 congenic mice were obtained from the Animal Production Area (NCI-Frederick, MD). Breeding pairs of B6 severe combined immunodeficient *scid/scid* (SCID) mice were generously provided by Dr. Robert H. Wiltrout (NCI-Frederick), and mice were bred in NCI-Frederick animal facility. All mice were kept in a specific pathogen free condition and used at 8-14 weeks of age.

Antibodies and generation of $F(ab')_2$ fragments. FITC-conjugated CD45.1, and CD45.2 were purchased from Pharmingen (San Diego, CA), and $F(ab')_2$ fragments of normal mouse IgG (NMG) were purchased from Jackson ImmunoResearch (West Grove, PA). $F(ab')_2$ fragments of anti-Ly49C and I (5E6, mouse IgG2a) and anti-Ly49G2 (4D11, rat IgG2a; the hybridoma was a gift from Dr. John Ortaldo, NCI-Frederick) monoclonal antibodies (mAb) were prepared as previously described [22]. The purity of $F(ab')_2$ fragments was determined by SDS-PAGE.

Cell lines. C1498 ($H2^b$), a murine leukemia, was obtained from American Type Culture Collection (Rockville, MD), and the bulk culture of C1498 were kept in log phase of growth for 7 days and frozen for later use. Frozen stocks were thawed every 2 months for in vitro assays. For in vivo experiments, frozen stocks were freshly thawed 7-10 days prior to in vivo administration and were kept in log phase of growth until use.

SCID NK cell culture. Splenocytes and BMC from B6 SCID mice were cultured in NK cell media (RPMI 1640 supplemented with 10% FBS, 100 u/ml penicillin/streptomycin, 50 μg/ml

gentamycin, 2 mM L-glutamine, 10 mM HEPES, 1mM non-essential amino acids, 1mM sodium pyruvate, $2.5x10^{-5}$ M 2-ME, and 1 μg/ml indomethacin) containing 5000 international units per ml (IU/ml) recombinant human IL-2 (rhIL-2; Developmental Therapeutics Program, NCI-Frederick) at 0.5 x 10^6 cells/ml for splenocytes or 1 x 10^6 cells/ml for BM cells for 5-7 days. These IL-2 activated SCID splenocytes and BMC were >97% $NK1.1^+$.

NK + BMC co-cultures and colony assays. Five x 10^5 B6 BMC were cultured with B6 SCID NK cells at NK to BMC ratio of 1:1, 1:5, or 1:10 in Iscove's Modified Dubecco's Medium (IMDM) supplemented with 10% FBS, $5x10^{-5}$M 2-ME, and 100 u/ml Penicillin/Streptomycin (10% IMDM) with 5000 IU/ml rhIL-2 in 24 well plates for 24 or 48 hours. In some experiments, 3 x 10^7 IL-2 activated NK cells were treated with 25 μg/ml of $F(ab')_2$ fragments of NMG, 5E6, or 4D11 for 2 hours in the presence of 5000 IU/ml rhIL-2 prior to co-culture with the equal number of BMC in T-25 flasks. The co-cultured cells were collected, and aliquots of BM cells were transferred into colony assay media [IMDM containing 25% FBS, 100 u/ml penicillin/streptomycin, 2mM L-glutamine, $5x10^{-5}$M 2-ME, 10 ng/ml recombinant murine (rm) GM-CSF (AMGen Corporation, Thousand Oakes, CA) and rmIL-3 (Developmental Therapeutics Program, NCI-Frederick), and 1.1% methyl cellulose (w/v)] so that 5 $x10^4$ BMC (based on the cell number at the initiation of the co-cultures) could be plated per 35 mm Petri dish in triplicates. Cultures were incubated for 7 days in humidified atmosphere at 37°C with 5% CO_2. At day 7, colonies were enumerated on a stereo microscope (Nikon, Melville, NY). The co-culture conditions were critical to obtain consistent results between in vitro colony assays and hematopoietic reconstitution in vivo.

Hematopoietic reconstitution studies. For experiments assessing the effects of NK cells on BMC, 3 to 6 x 10^7 B6 SCID NK cells were pre-incubated with 300-500 μg of $F(ab')_2$ fragments of NMG, 5E6, or 4D11 in 2-3 ml 10% IMDM at 37°C for 2-3 hours in the presence of 5000 IU/ml rhIL-2. BMC were prepared from B6 or B6.Ly5.2 mice and cultured either alone or with the NK cells at BMC:NK ratio of 1:1 in 10% IMDM in the presence of 5000 IU/ml rhIL-2 for 24 or 48 hours. The co-cultured cells were then harvested and resuspended in Dulbecco's Phosphate Buffered Saline (DPBS) to be used in the colony assay as described above and for injection of the animals. B6 recipients were lethally irradiated at 8.5 Gy and infused with the cells at 3-5x10^6 BMC and 3-5x10^6 NK per mouse (i.v.) based on the cell numbers at the initiation of the co-culture. At various days post-BMT, splenocytes and BMC were prepared from 3-4 mice per group and were used in colony assays to determine myeloid reconstitution as described above. Peripheral blood samples were collected in EDTA-treated Microtainer tubes (Becton Dickinson, San Jose, CA) red blood cell (RBC) and white blood cell (WBC) counts were determined by analyzing the blood samples on HEMAVET Multispecies Hematology Analyzer (CDC Technologies). In the experiments using B6.Ly5.2 congenic mice as donors, the level of donor chimerism was determined at day 98 post-BMT by staining splenocytes and BMC with FITC-conjugated anti-mouse CD45.1 or CD45.2 and analyzing on FACScan (Becton Dickinson, San Diego, CA).

Ex-vivo purging of tumor and survival studies. For purging of tumor cells, 2 x 10^7 or 3 x 10^7 B6 SCID NK cells were pre-treated with 300 μg of 5E6 or 4D11 $F(ab')_2$ for 2-3 hours. NK cells were cultured further with 3 x 10^7 B6 BMC and 2 x 10^5 or 3 x 10^5 C1498 (NK:BMC = 1:1 or less and NK:C1498 = 100:1) for 24 hours. The co-cultured cells were then harvested and

injected into lethally irradiated (8.5 Gy) B6 recipient mice (8-10 mice per group per experiment) at 3 x 10^6 BMC, 2-3 x 10^6 NK, and 2-3 x 10^4 C1498 cells per mouse (i.v.) based on the cell numbers at the initiation of co-cultures. Mice were then monitored for survival. Additionally, aliquots of the co-cultured cells were transferred into colony assay media without rmGM-CSF and rmIL-3 for colony assay as described above.

Statistics. Nonparametric analysis of oneway variation (ANOVA) with Newman-Keuls test was used for in vitro assays and log-rank test was used for survival studies.

RESULTS

NK cells can exert inhibitory effects on BMC during co-cultures.

While exposure of tumor cells contaminating the bone marrow to high numbers of activated NK cells would be more effective to eliminate the tumor cells, NK cells may have deleterious effects on BMC because of cytokines, such as IFN-γ, that have been shown to inhibit growth of BMC in some in vitro studies [2,30]. In order to determine under which condition NK cells alone can have inhibitory effects on syngeneic BMC, B6 BMC were co-cultured with IL-2 activated NK cells derived from B6 SCID mice at NK to BMC ratios (NK:BMC) of 10, 5, or 1 to 1 for 24 hours. Co-cultured cells then were transferred into semi-solid media containing rmGM-CSF and rmIL-3 and cultured further for 7 days. As shown in Figure 1A, the growth of granulocyte/monocyte progenitors (CFU-GM) was significantly inhibited in the co-cultures with higher NK:BMC ratios as compared to BMC control that were cultured alone such that no colonies could be detected in the co-cultures with NK:BMC = 10:1. However, the number of CFU-GM from the co-cultures of NK:BMC = 1:1 was not affected in 24 hours as compared to the BMC only control (Figure 1A and B). Since an optimal condition for purging of tumor cells contaminating BMC may require co-culture with NK cells longer than 24 hours, the effect of NK cells on BMC at NK:BMC = 1:1 was further examined in 48 hour co-culture studies. The results show that growth of granulocyte/monocyte progenitors was significantly but not completely inhibited by NK cells as compared to the BMC only controls in 48 hour co-cultures (Figure 1C) whereas it was not affected in 24 hours (Figure 1A and B). BMC derived from B6 mice express MHC class I molecule, $H2K^b$, that is a specific ligand for inhibitory receptors Ly49C and I present on a subset of NK cells. Cross-linking of the inhibitory receptors by these class I ligands

present on tumor cells may inactivate the subset of NK cells expressing Ly49C and I. We have previously shown that blockade of Ly49C and I with $F(ab')_2$ fragments of 5E6 mAb specific for the receptor augments anti-tumor effects in vitro [29]. However, by this same mechanism, blockade of NK inhibitory receptors to self MHC molecules may increase autoreactivity to hematopoietic cells. To assess if blockade of NK inhibitory receptors has any adverse effects on BMC, IL-2 activated B6 SCID NK cells were pre-treated with media or $F(ab')_2$ fragments of NMG, 5E6 or 4D11 mAbs. 4D11 is a mAb specific for Ly49G2 receptor, which is expressed on 50-60% of NK cells and binds to MHC $H2D^d$ [22] but not to $H2K^b$ (Table 1) and thus was used as an irrelevant antibody control in the co-cultures of NK and BMC derived from B6 ($H2^b$) mice. Pre-treated NK cells were then cultured with BMC for 24 or 48 hours, after which survival of hematopoietic progenitors was assessed in colony assays. The results indicate that blockade of neither Ly49C and I nor Ly49G2 inhibitory receptors during the 24 hour co-cultures suppressed the level of CFU-GM compared to BMC control cultured with NMG $F(ab')_2$-treated NK cells (Figure 1B). Similarly, treatment of NK cells with 5E6 or 4D11 $F(ab')_2$ fragments did not result in further suppression of granulocyte/monocyte growth in 48 hours as compared to the control co-cultures (Figure 1C) suggesting that blockade of inhibitory signals in NK+BMC co-cultures does not decrease the growth of granulocyte/monocyte progenitors to a greater extent than the inhibition mediated by untreated NK cells in vitro.

Inhibitory receptor blockade during 24 hour NK+BMC co-culture does not affect hematopoietic reconstitution in vivo.

Although no deleterious effects of NK inhibitory receptor blockade on BMC were observed in a short-term in vitro assay examining the growth of granulocyte/monocyte

progenitors, NK cells may have affected the ability of BMC to reconstitute in vivo after BMT. Therefore, we examined the ability of BMC that were co-cultured with NK cells with or without inhibitory receptor blockade to repopulate lethally irradiated syngeneic recipients after BMT. Additionally, to assess the long-term donor chimerism after syngeneic BMT, we utilized BMC from B6 mice that are congenic for Ly5.2 (CD45.1) antigen expression, which can be distinguished from Ly5.1 (CD45.2) antigen expressed by the conventional host B6 mice by flow cytometric analysis. B6.Ly5.2 BMC were cultured either alone or with activated B6 SCID NK cells pre-treated with $F(ab')_2$ fragments of NMG, 5E6, or 4D11 at NK:BMC=1:1 for 24 hours, and lethally irradiated B6 recipient mice were infused with the co-cultured BMC. At days 7, 14, 21, and 27 post-BMT, the level of myeloid reconstitution was determined using splenocytes and BMC in colony assays. The results indicate that neither NK cells alone nor those with blockade of Ly49C and I inhibitory receptors had any adverse effects on myeloid reconstitution in spleen and bone marrow as compared to the control mice at all time points post-BMT (Figure 2). Additionally, there was no difference in reconstitution of mature RBC and WBC as determined by RBC and WBC counts in peripheral blood (data not shown). In order to determine the effect of Ly49C and I receptor blockade on long-term donor chimerism, splenocytes from the recipient mice were analyzed for the levels of donor cell engraftment as demonstrated by Ly5.2 antigen expression at day 98 post-BMT. The levels of donor chimerism were comparable in all recipients regardless the co-culture conditions (Table 2) demonstrating that the ability of BMC to reconstitute lethally irradiated hosts is not affected by blockade of NK inhibitory receptors during 24 hour co-culture period.

Suppressive effects of NK cells on BMC in 48 hour co-cultures result in short-term inhibition of myeloid reconstitution in vivo.

In contrast to the 24 hour co-cultures of NK and BMC, culturing of BMC with NK cells for 48 hours resulted in a a significant decrease in the numbers of CFU-GM compared to the control in vitro ($p<0.01$, Figure 1C). Thus, whereas BMC in the 24 hour co-cultures were not altered in their ability to reconstitute lethally irradiated syngeneic recipients, the adverse effects of NK cells on BMC observed in 48 hour co-cultures may result in suppression of hematopoietic reconstitution in vivo. To address this, NK cells were pre-treated with the $F(ab')_2$ fragments of NMG, 5E6, or 4D11 and co-cultured with BMC for 48 hours. Lethally irradiated B6 mice were then injected with the co-cultured cells, and at days 7, 14, 21, and 28 post-BMT, the effects on myeloid reconstitution of the recipients were examined. The results show that the levels of myeloid reconstitution were significantly decreased in spleen ($p<0.001$, Figure 3) and the bone marrow ($p<0.001$ Figure 3) of mice receiving NK+BMC co-cultures at day 7 post-BMT as compared to the control mice receiving BMC cultured alone. The extent of suppression of myeloid reconstitution at day 7 post-BMT was not significantly different among the treatment groups correlating with the in vitro assays (Figure 1C). At day 14 post-BMT, the suppression of myeloid reconstitution in mice receiving BMC cultured with NMG $F(ab')_2$- or 4D11 $F(ab')_2$-treated NK cells was normalized such that in spleen, the level of myeloid reconstitution was comparable to the control mice transplanted with BMC cultured alone (Figure 3). More importantly, myeloid reconstitution was significantly suppressed in both spleen ($p<0.01$) and the bone marrow ($p<0.001$) of only those mice receiving BMC cultured with NK cells that were pre-incubated with 5E6 $F(ab')_2$ and not with 4D11 $F(ab')_2$ (Figure 3) suggesting that even though further inhibition of CFU-GM growth due to inhibitory receptor blockade was not observed in

vitro, the ability of hematopoietic precursors to reconstitute lethally irradiated recipients was suppressed by inhibition of NK inactivation by the receptor blockade. However, the results indicate that the inhibition of myeloid reconstitution was transient in that by day 28 post-BMT, the levels of CFU-GM were comparable among all groups (Figure 4). Assessment of reconstitution of peripheral blood cells indicates that while NK cells exerted suppressive effects on neutrophil and platelet recovery transiently at day 14 post-BMT, inhibitory receptor blockade did not result in additional inhibition (Figure 5).

Blockade of Ly49C and I receptors augments purging effects of NK cells against contaminating tumor cells in $H2^b$ mice.

Since activated NK cells, with or without inhibitory receptor blockade, did not have any deleterious effects on BMC in 24 hour co-cultures at NK:BMC = 1:1 in vitro as well as in vivo, blockade of NK inhibitory receptors under the same conditions may result in increased anti-tumor effects against syngeneic tumor cells without affecting BMC. To determine whether NK cell-mediated purging of tumor is enhanced under these co-culture conditions, IL-2 activated B6 SCID NK cells were pre-treated with $F(ab')_2$ fragments of 5E6 or 4D11 and co-cultured with B6 BMC (NK:BMC=1:1) contaminated with C1498 leukemia cells (NK:tumor=100:1) for 24 hours. The cells from the co-cultures were used in colony forming assays to assess the effects of Ly49C and I blockade on anti-tumor activity. As shown in Figure 6A, the growth of C1498 tumor cells was significantly decreased in the presence of NK cells treated with either antibody in 24 hours ($p<0.0001$) as compared to the tumor only control. Moreover, greater inhibition of the tumor cell growth was observed when NK cells were treated with 5E6 $F(ab')_2$, and this effect was 5E6 $F(ab')_2$ specific as demonstrated by a lack of such inhibition when 4D11 $F(ab')_2$-treated NK cells

were used in the co-cultures ($p<0001$, Figure 6A). When the co-cultured cells were used in BMT of lethally irradiated B6 mice, the groups injected with C1498 contaminated BMC cultured with the control antibody, 4D11 $F(ab')_2$-treated NK cells survived significantly longer than the BMC and tumor only control group ($p<0.0001$, Figure 6B). More importantly, mice receiving tumor contaminated BMC cultured with 5E6 $F(ab')_2$-treated NK cells survived significantly longer than those receiving tumor contaminated BMC cultured with NK cells and the control 4D11 $F(ab')_2$ fragments ($p=0.0417$, Figure 6B). Surviving mice had normal hematopoietic reconstitution as measured by various hematopoietic parameters (data not shown). These results demonstrate that under appropriate conditions, blockade of inhibitory receptors suppresses the inactivation of NK cells and augments anti-tumor activity without long-term deleterious effects on myeloid reconstitution after BMT.

DISCUSSION

The use of autologous BMT as a therapy for cancer is limited due to a higher relapse rate that can result from tumor cells contaminating the bone marrow [7,8]. Previous studies have demonstrated the potential of activated autologous NK cells for purging of the contaminating tumors prior to BMT to improve its efficacy [2,3]. However, the purging effect mediated by autologous NK cells may be hampered by the inhibitory receptors expressed on the subset of NK cells [14,16]. The missing self hypothesis states that NK cells are in activated state unless they interact with the self MHC class I, and inactivation of NK cell function via negative signals results from the interaction between inhibitory receptors and MHC class I determinants specific for the receptors [31]. Expression of at least one inhibitory receptor specific for self MHC class I may prevent NK cells from autoreactivity but also minimizes cytotoxicity against the tumor cells that express the same determinant or ligands for activating receptors. In this study, we show that under certain conditions, blockade of NK cell inhibitory receptors augments purging effects against syngeneic tumor cells contaminating BMC without inducing significant autoreactivity to "self" hematopoietic cells (Figure 2). However, inhibitory receptor blockade can result in significant suppression of syngeneic BMC depending on the conditions used for NK+BMC co-cultures (Figure 3).

Previous studies indicated that NK cells alone can have different effects on BMC depending on the conditions in which the BMC are placed. If the culture conditions are suboptimal for hematopoietic progenitor growth, then the cytokines such as GM-CSF, G-CSF, and IL-1β produced by activated NK cells can promote the growth of BMC [12,30]. However, if the culture conditions are optimal for growth of hematopoietic progenitors, then NK cells mediate inhibitory effects partially via the production of IFN-γ [12]. This is consistent with the

increased suppression of the level of CFU-GM in the presence of increasing numbers of NK cells (Figure 1). In addition, the results demonstrate that the duration of exposure of BMC to syngeneic NK cells can determine the extent of inhibition on BMC since the growth of CFU-GM in vitro was suppressed after 48 hour co-culture even at an NK to BMC ratio that had no inhibitory effect in 24 hours (Figure 1). Similarly, NK cell-mediated suppressive effects on myeloid progenitors due to inhibitory receptor blockade could be observed in vivo, albeit transiently, only if NK and BMC co-cultured with 5E6 $F(ab')_2$ fragments for 48-hours, but not 24 hours, were used in BMT (Figure 2 and 3). Therefore, the results indicate that a balance between these factors is essential to optimize the hematopoietic recovery with effective abrogation of tumor cells contaminating the bone marrow.

It is interesting that while in vitro growth of CFU-GM from BMC co-cultured with 5E6 $F(ab')_2$-treated NK cells for 48-hours was not inhibited as compared to that from controls co-cultured with NMG $F(ab')_2$- or 4D11 $F(ab')_2$-treated NK cells (Figure 1C), myeloid reconstitution of mice transplanted with these co-cultures demonstrated impaired reconstitution only in the group which received BMC co-cultured with 5E6 $F(ab')_2$-treated NK cells (Figure 3). Moreover, 5E6 $F(ab')_2$ treatment resulted in suppression of early myeloid reconstitution without affecting mature hematopoietic parameters in the peripheral blood (Figure 4). It may be that the blockade of inhibitory receptors allows NK cells to exert greater suppressive effects selectively on myeloid progenitors. Presumably, every NK cell has at least one inhibitory receptor to self MHC class I to prevent autoreactivity, but in the absence of inhibitory signal, an activating receptor, which has been shown to play a critical role in NK response to MCMV infection [32-34], may play a role in the selective myelosuppression. The mechanism of NK cell-mediated suppressive effects due to inhibitory receptor blockade is yet to be defined; it may be due to

direct cytotoxicicity against myeloid progenitors or indirect effects by increased production of inhibitory cytokines (i.e. IFN-γ) [24,25]. While the NK cell-mediated suppressive effects on myeloid progenitors is transient in this model in which total BMC are used, the selective inhibition of myeloid reconstitution due to inhibitory receptor blockade may be more severe, even in a shor-term co-culture period, if a purified population such as $CD34^+$ cells [3,6] are used in the purging process and BMT.

We have recently reported that adoptive transfer of activated NK cells treated with 5E6 $F(ab')_2$ ex vivo into C1498 tumor bearing mice significantly increases the rate of survival compared to the control treated mice [29]. The current study further demonstrates that blockade of Ly49C and I inhibitory receptors with 5E6 $F(ab')_2$ during in vitro purging of C1498 leukemia contaminating BMC significantly augments the anti-tumor effects mediated by NK cells. In addition, the results indicate that the enhanced anti-tumor effects can be achieved without inducing significant autoreactivity as evidenced by myelosuppression. The reduction in tumor growth in vitro and the increase in the survival of mice transplanted with purged BMC in the presence of 5E6 $F(ab')_2$, but not 4D11 $F(ab')_2$, the irrelevant antibody control specific for Ly49G2 present on 50-60% of NK cells but does not interfere with binding of $H2^b$ (Figure 6), were observed under the same co-culture condition in which no adverse effects on hematopoietic reconstitution resulted in vivo.

The suppressive effect of NK inhibitory receptor blockade during 48-hour co-culture was transiently and did not affect reconstitution of peripheral blood cells. Thus, variations of purging conditions such as prolonging the period of purging or blockade of multiple inhibitory receptors specific for self MHC class I to eradicate resistant tumor cells should be attempted with caution to determine if the benefits of increased anti-tumor activity outweigh the potential harmful

effects on hematopoietic reconstitution. Taken together, this study demonstrates that blockade of inhibitory receptors on activated NK cells during in vitro purging of syngeneic tumors and after BMT with the in vitro purged BMC will provide another avenue to increase anti-tumor activity mediated by NK cells without significant autoreactivity. Therefore, NK inhibitory receptor blockade can be of potential use to increase the efficacy of autologous BMT as a means of cancer therapy.

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Table 1. Ly49 family of receptors and the ligands

Category	Receptors	Ligands	mAb[a]
Inhibitory	Ly49A	$H2D^d$	YE1/32
	Ly49C/I	$H2K^b$	5E6
	Ly49G2	$H2D^d$	4D11
Activating	Ly49D	$H2D^d$	4E5
	Ly49H	?	1F8

[a]Monoclonal antibodies specific for Ly49 receptors.

Table 2. Effect of Ly49C and I receptor blockade on expression of Ly5.2 antigen in spleen.

Treatment[a]	No. of Mice	% Donor Chimerism[b]
No NK	3	95 ± 0.4[c]
NK+NMG	3	94 ± 0.4
NK+5E6	3	94 ± 0.3
NK+4D11	3	95 ± 0.2

[a]B6 SCID NK cells were pre-treated with $F(ab')_2$ fragments of NMG, 5E6, or 4D11 mAbs prior to the co-cultures with donor, B6.Ly5.2 BMC.

[b]At day 98 post-BMT splenocytes from mice were incubated with either FITC-anti-Ly5.1 (host) or anti-Ly5.2 (donor) mAb and analyzed on FACScan.

[c]The numerical values represent $Ly5.2^+$ cells as % lymphocytes ± SEM.

FIGURE LEGENDS

Figure 1. Effect of NK cells and inhibitory receptor blockade on syngeneicBMC in vitro. Five x 10^5 B6 BMC were cultured with IL-2 activated B6 SCID NK cells at NK to BMC ratio of 1:1, 1:5, or 1:10 in 10% IMDM containing 5000 IU/ml rhIL-2 in 24 well plates for 24 hours (A). Three x 10^7 IL-2 activated NK cells were treated with 25 μg/ml of $F(ab')_2$ fragments of NMG, 5E6, or 4D11 for 2 hours in the presence of 5000 IU/ml rhIL-2 prior to co-culture with 3 x 10^7 BMC (NK:BMC=1:1) in T-25 flasks for 24 (B) or 48 (C) hours. The co-cultured cells were collected, and aliquots of BMC were transferred into colony assay media containing 1.1% methyl cellulose and 10 ng/ml rmGM-CSF and rmIL-3 so that 5 x10^4 BMC (based on the cell number at the initiation of the co-cultures) could be plated per 35 mm Petri dish in triplicates. The cells were incubated for 7 days in humidified atmosphere at 37°C with 5% CO_2 and number of colonies were counted. Mean values of the triplicates ± SEM from a representative of 3 (A and B) or 4 (C) independent experiments are shown. Non-parametric ANOVA with Newman-Keuls multiple group comparison test was used for statistical analysis.

Figure 2. Effect of inhibitory receptor blockade during 24 hour co-culture on myeloid reconstitution after BMT. Three x 10^7 B6 SCID NK cells were pre-incubated with 300 μg of $F(ab')_2$ fragments of NMG, 5E6, or 4D11 in 10% IMDM at 37°C for 2-3 hours in the presence of 5000 IU/ml rhIL-2. BMC were prepared from B6.Ly5.2 mice and cultured either alone or with the NK cells at BMC:NK ratio of 1:1 in 10% IMDM in the presence of 5000 IU/ml rhIL-2 for 24 hours. At 24 hours, B6 recipients were lethally irradiated at 8.5 Gy and infused with the co-cultured cells at 3x10^6 BMC and 3x10^6 NK per mouse (i.v.) based on the cell numbers at the

initiation of the co-culture. At various days post-BMT, colony assays were performed using splenocytes and BMC (4 mice/group) to determine myeloid reconstitution in spleen (A) and bone marrow (B). A representative of 2 independent experiments is shown as mean ± SEM. Non-parametric ANOVA with Newman-Keuls multiple group comparison test was used for statistical analysis and the mean values of the various groups were not significantly different ($p>0.05$).

Figure 3. Effect of inhibitory receptor blockade during 48 hour co-culture on myeloid reconstitution at days 7 and 14 post-BMT. Six x 10^7 B6 SCID NK cells were pre-incubated with 500 μg of $F(ab')_2$ fragments of NMG, 5E6, or 4D11 in 10% IMDM at 37°C for 2-3 hours in the presence of 5000 IU/ml rhIL-2. BMC were prepared from B6 mice and cultured either alone or with the NK cells at BMC:NK ratio of 1:1 in 10% IMDM in the presence of 5000 IU/ml rhIL-2 for 48 hours. At 48 hours, B6 recipients were lethally irradiated at 8.5 Gy and infused with the co-cultured cells at $5x10^6$ BMC and $5x10^6$ NK per mouse (i.v.) based on the cell numbers at the initiation of the co-culture. At days 7 and 14 post-BMT, colony assays were performed using splenocytes and BMC (3-4 mice/group) to determine myeloid reconstitution in spleen and bone marrow. A representative of 3 independent experiments is shown as mean ± SEM. Non-parametric ANOVA with Newman-Keuls multiple group comparison test was used for statistical analysis.

Figure 4. Effect of inhibitory receptor blockade during 48 hour co-culture on myeloid reconstitution after BMT. Six x 10^7 B6 SCID NK cells were pre-incubated with 500 μg of $F(ab')_2$ fragments of NMG, 5E6, or 4D11 in 10% IMDM at 37°C for 2-3 hours in the presence of 5000 IU/ml rhIL-2. BMC were prepared from B6 mice and cultured either alone or with the NK

cells at BMC:NK ratio of 1:1 in 10% IMDM in the presence of 5000 IU/ml rhIL-2 for 48 hours. At 48 hours, B6 recipients were lethally irradiated at 8.5 Gy and infused with the co-cultured cells at $5x10^6$ BMC and $5x10^6$ NK per mouse (i.v.) based on the cell numbers at the initiation of the co-culture. At various days post-BMT, colony assays were performed using splenocytes and BMC (3-4 mice/group) to determine myeloid reconstitution in spleen and bone marrow. A representative of 3 independent experiments is shown as mean ± SEM. Non-parametric ANOVA with Newman-Keuls multiple group comparison test was used for statistical analysis.

Figure 5. Effect of inhibitory receptor blockade during 48 hour co-culture on recovery of red blood cells and leukocytes after BMT. Six x 10^7 B6 SCID NK cells were pre-incubated with 500 μg of $F(ab')_2$ fragments of NMG, 5E6, or 4D11 in 10% IMDM at 37°C for 2-3 hours in the presence of 5000 IU/ml rhIL-2. BMC were prepared from B6 mice and cultured either alone or with the NK cells at BMC:NK ratio of 1:1 in 10% IMDM in the presence of 5000 IU/ml rhIL-2 for 48 hours. At 48 hours, B6 recipients were lethally irradiated at 8.5 Gy and infused with the co-cultured cells at $5x10^6$ BMC and $5x10^6$ NK per mouse (i.v.) based on the cell numbers at the initiation of the co-culture. At various days post-BMT, peripheral blood was collected (3-4 mice/group) in EDTA-treated Microtainer tubes and analyzed by HEMAVET multispecies hematology analyzer to determine the neutrophil (A), RBC (B), and platelet (C) counts. A representative of 2 independent experiments is shown as mean ± SEM. Non-parametric ANOVA with Newman-Keuls multiple group comparison test was used for statistical analysis.

Figure 6. Effect of blockade of Ly49C/I receptors on purging C1498. Three x 10^7 IL-2 activated B6 SCID NK cells were pre-treated with 300 μg of 5E6 or 4D11 $F(ab')_2$ for 2-3 hours.

NK cells were cultured further with 3 x 10^7 B6 BMC and 3 x 10^5 C1498 (NK:BMC = 1:1 and NK:C1498 = 100:1) for 24 hours. At 24 hours, aliquots of the co-cultured cells were transferred into colony assay media without rmGM-CSF and rmIL-3 so that 100 or 1000 C1498 cells (based on the cell number at the initiation of the co-culture) are plated per Petri dish in triplicate to enumerate C1498 colonies in vitro (A). A representative of 2 independent experiments is shown as mean ± SEM, and non-parametric ANOVA with Newman-Keuls multiple group comparison test was used for statistical analysis. Lethally irradiated (8.5 Gy) B6 mice were injected with the co-cultured cells at 3 x 10^6 BMC, 3 x 10^6 NK, and 3 x 10^4 C1498 cells per mouse (i.v.) based on the cell numbers at the initiation of co-cultures and were monitored for survival (B). Pooled data from 2 independent experiments is shown (n=18 per group), and log-rank test was used for statistical analysis.

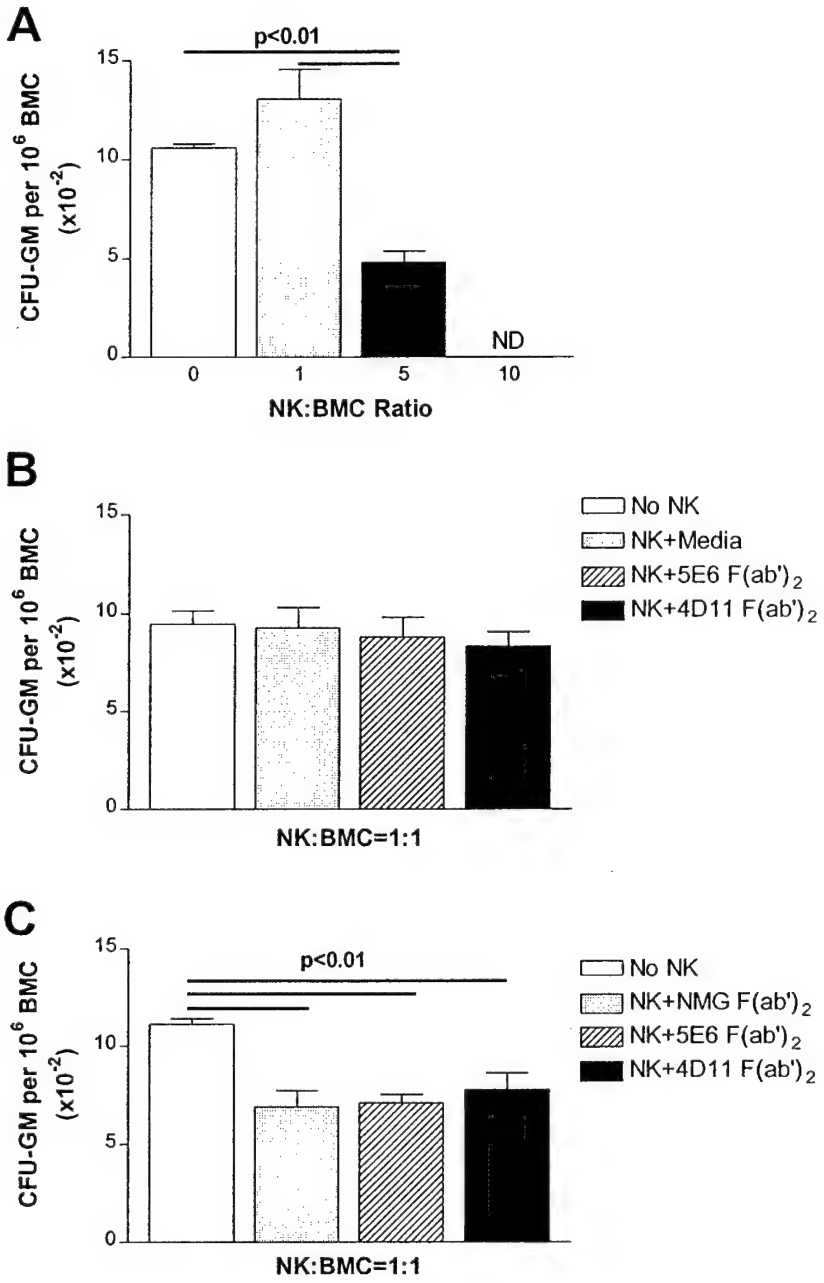
A
p<0.01
CFU-GM per 10^6 BMC (x10^-2)
15
10
5
0
ND
0
1
5
10
NK:BMC Ratio
B
No NK
NK+Media
NK+5E6 F(ab')2
NK+4D11 F(ab')2
CFU-GM per 10^6 BMC (x10^-2)
NK:BMC=1:1
C
p<0.01
No NK
NK+NMG F(ab')2
NK+5E6 F(ab')2
NK+4D11 F(ab')2
CFU-GM per 10^6 BMC (x10^-2)
NK:BMC=1:1

Figure 1

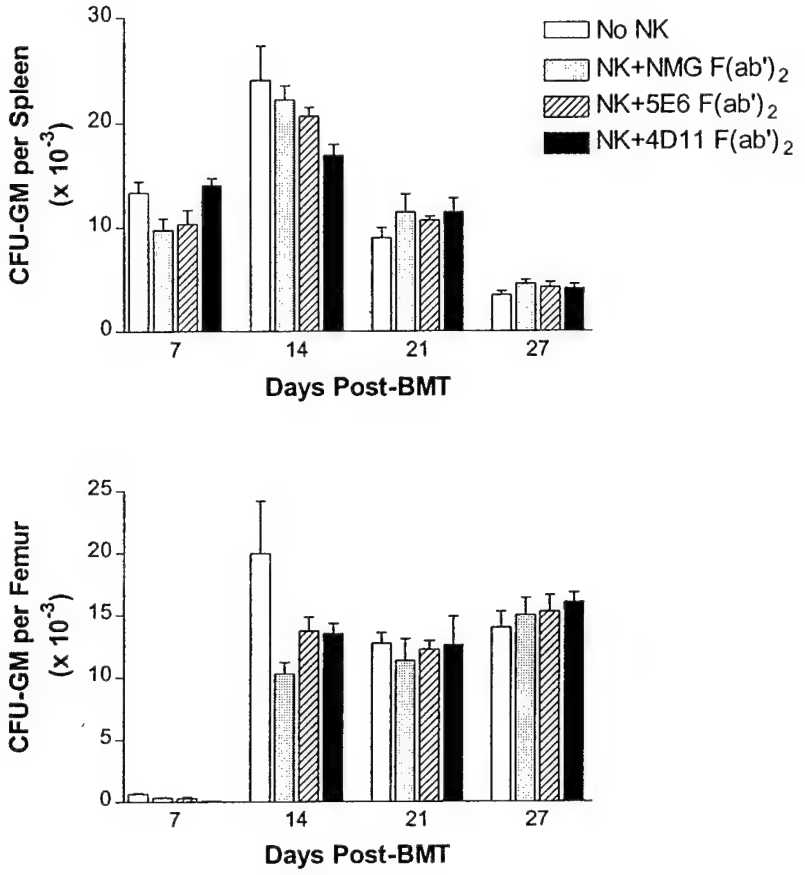
No NK
NK+NMG F(ab')2
NK+5E6 F(ab')2
NK+4D11 F(ab')2
CFU-GM per Spleen (x 10-3)
30
20
10
0
7
14
21
27
Days Post-BMT
CFU-GM per Femur (x 10-3)
25
20
15
10
5
0
7
14
21
27
Days Post-BMT

Figure 2

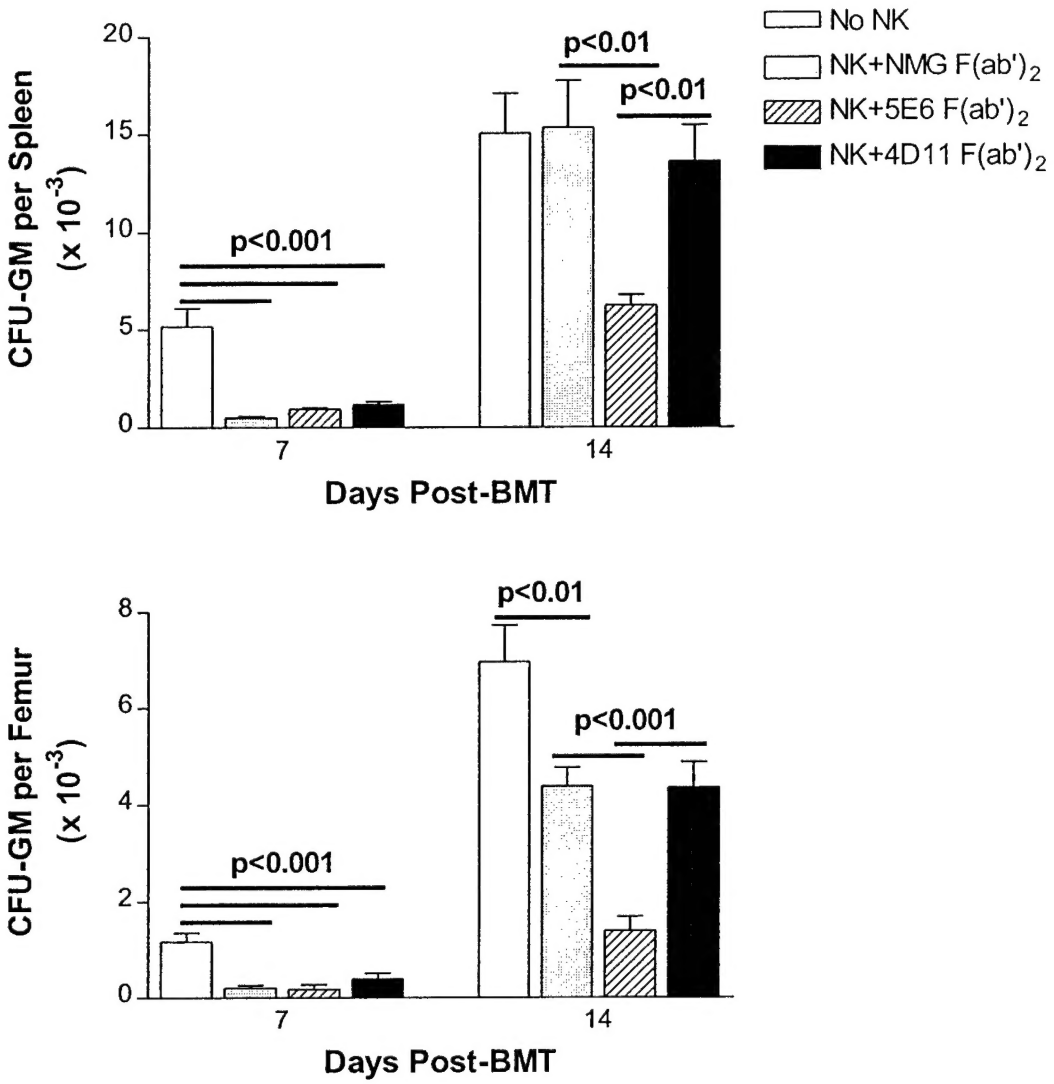
No NK
NK+NMG F(ab')2
NK+5E6 F(ab')2
NK+4D11 F(ab')2
CFU-GM per Spleen (x 10-3)
20
15
10
5
0
p<0.01
p<0.01
p<0.001
7
14
Days Post-BMT
CFU-GM per Femur (x 10-3)
8
6
4
2
0
p<0.01
p<0.001
p<0.001
7
14
Days Post-BMT

Figure 3

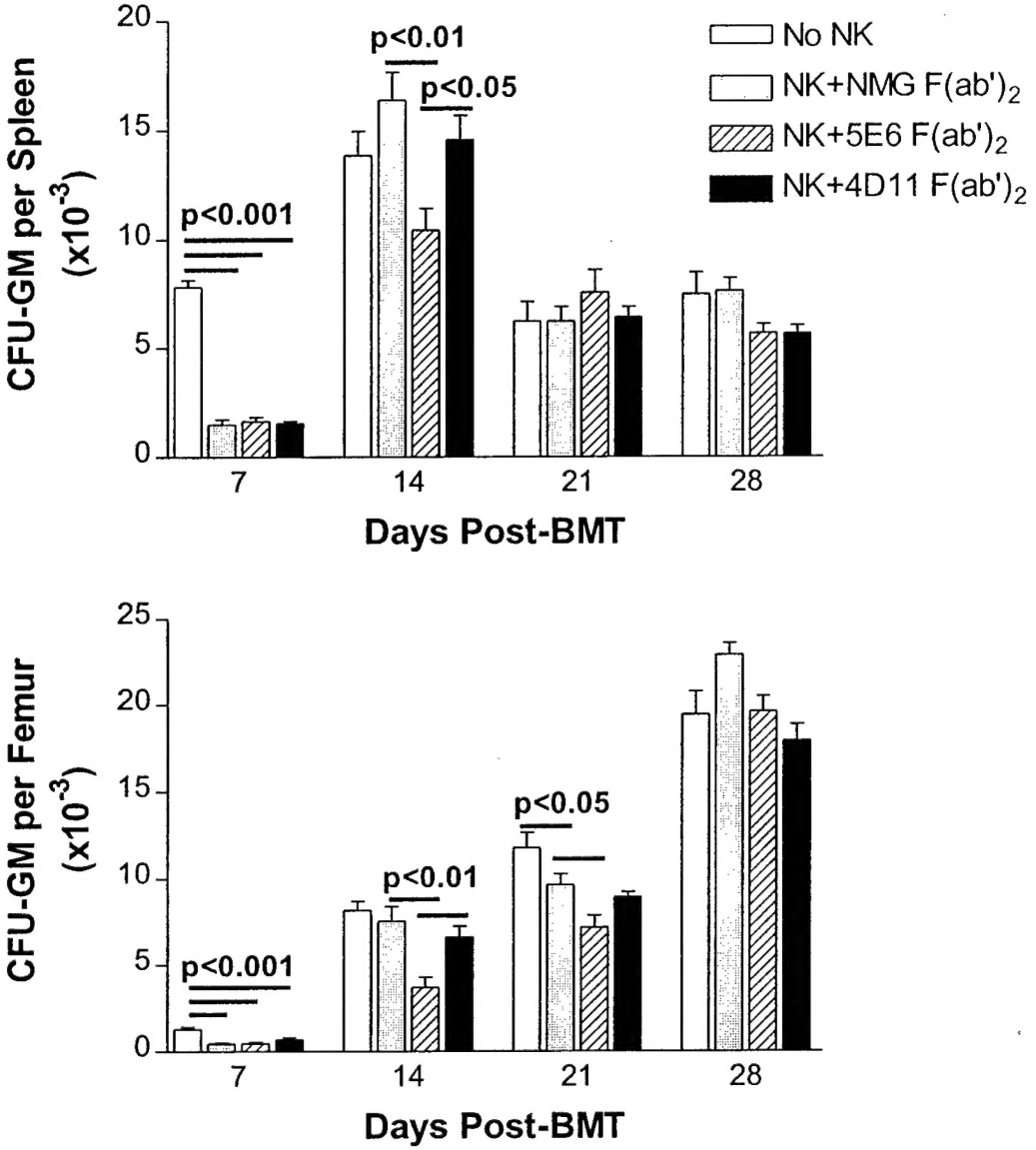
CFU-GM per Spleen (x10^{-3})
20
15
10
5
0
p<0.01
p<0.05
p<0.001
No NK
NK+NMG F(ab')$_2$
NK+5E6 F(ab')$_2$
NK+4D11 F(ab')$_2$
7
14
21
28
Days Post-BMT
CFU-GM per Femur (x10^{-3})
25
20
15
10
5
0
p<0.05
p<0.01
p<0.001
7
14
21
28
Days Post-BMT

Figure 4

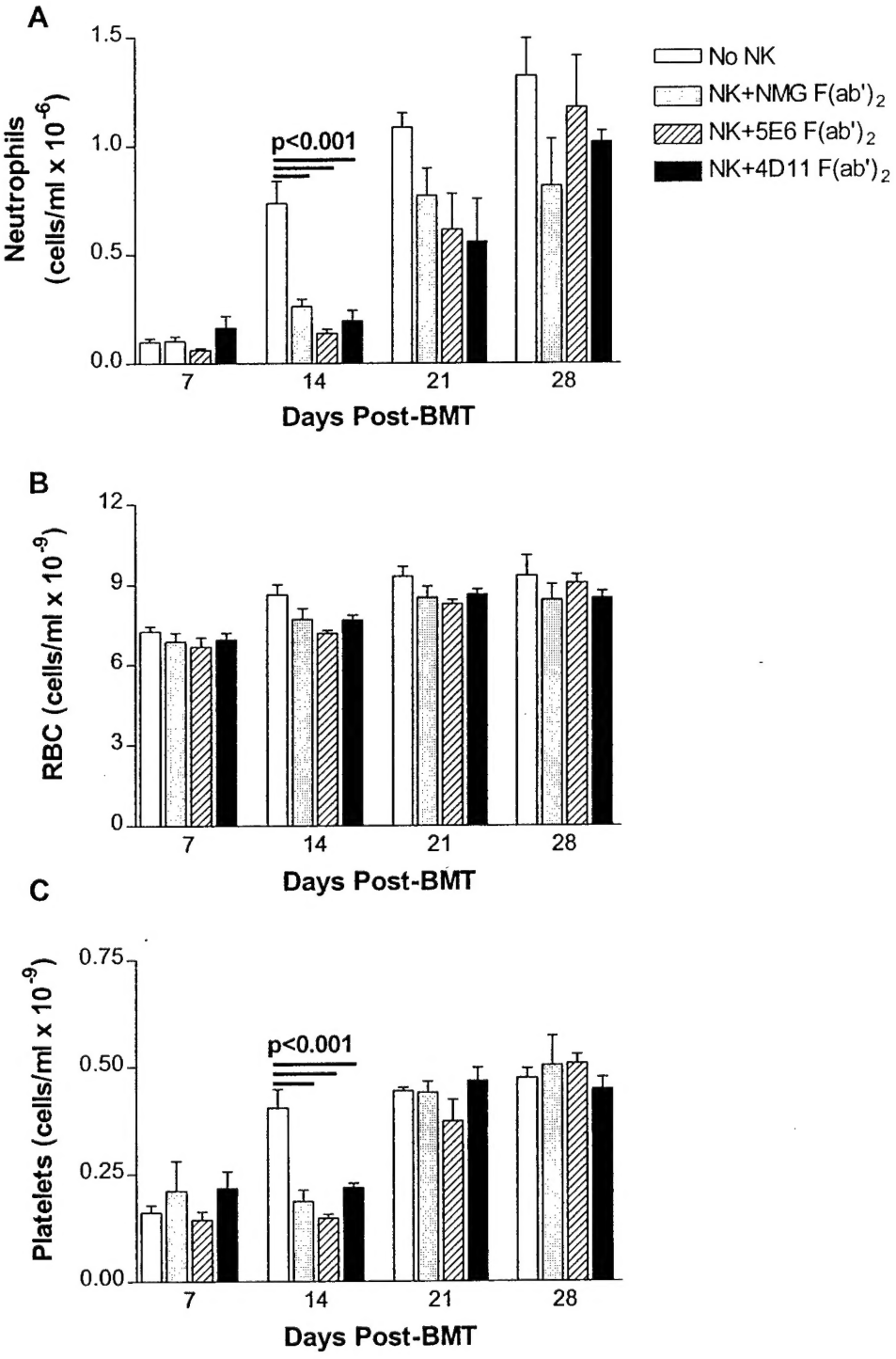
A
Neutrophils (cells/ml x 10^{-6})
1.5
1.0
0.5
0.0
p<0.001
7
14
21
28
Days Post-BMT
No NK
NK+NMG F(ab')$_2$
NK+5E6 F(ab')$_2$
NK+4D11 F(ab')$_2$
B
RBC (cells/ml x 10^{-9})
12
9
6
3
0
7
14
21
28
Days Post-BMT
C
Platelets (cells/ml x 10^{-9})
0.75
0.50
0.25
0.00
p<0.001
7
14
21
28
Days Post-BMT

Figure 5

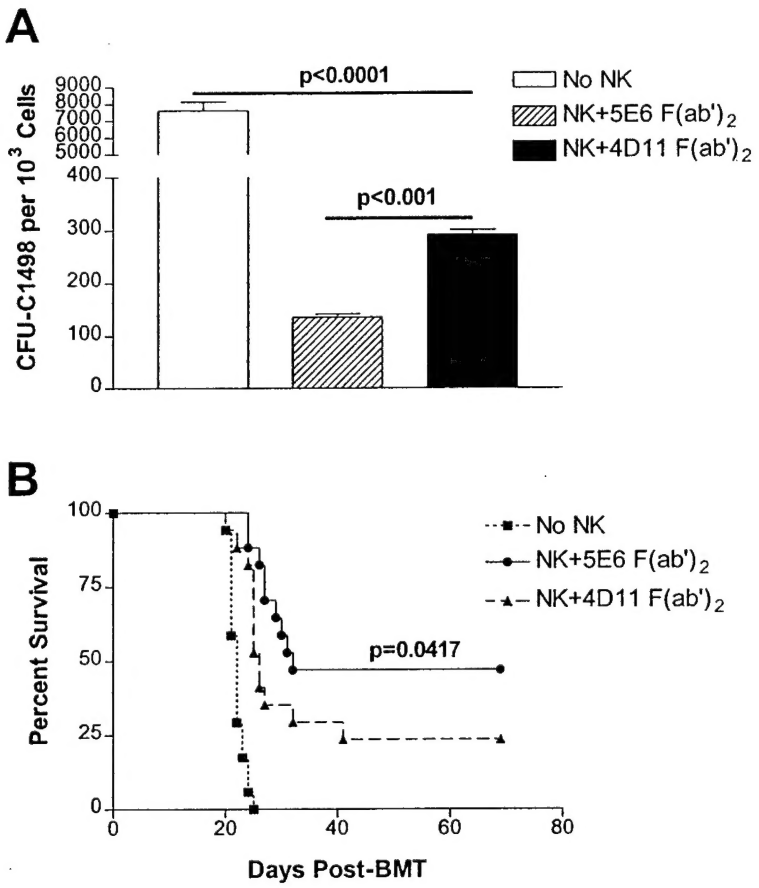
A
p<0.0001
No NK
NK+5E6 F(ab')2
NK+4D11 F(ab')2
p<0.001
CFU-C1498 per 10^3 Cells
9000
8000
7000
6000
5000
400
300
200
100
0
B
No NK
NK+5E6 F(ab')2
NK+4D11 F(ab')2
p=0.0417
Percent Survival
100
75
50
25
0
0
20
40
60
80
Days Post-BMT

Figure 6